THE TOXICITY OF THE ORGANOPHOSPHATE INSECTICIDE FENITROTHION AND ITS METABOLISM BY BLUE CRABS, *CALLINECTES SAPIDUS*

BY

JOHN J. JOHNSTON

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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JOHN J. JOHNSTON

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The LC_{50} for Callinectes sapidus exposed to fenitrothion for 96 hours at 22°C and a salinity of 34 ppt (parts per thousand) was estimated to be 8.6 µg/liter. Acute toxicity was shown to decrease with decreasing salinity and decreasing temperature. Exposure to a simulated tidal cycle increased the acute toxicity of fenitrothion to Callinectes. The autotomization response in Callinectes was shown to be affected at subacute exposure levels as low as 0.1 µg/liter. In vitro studies showed that the rates of formation of 3-methyl-4-nitrophenol and desmethyl fenitrothion were greater and the rate of formation of fenitrooxon was less in subcellular fractions prepared from the hepatopancreas of crabs which had been acclimated to lower salinity seawater. All three of these metabolites were formed at faster rates in subcellular fractions prepared from crabs which had been acclimated to and incubated at 22°C than at 17°C. Tissue

distribution of aniline hydroxylase and glutathione-S-transferase activity was also determined. The uptake of ^{14}C-fenitrothion at a level in seawater of 5.2 μg/liter was greater at 22°C than at 17°C and from 34 ppt than from 17 ppt seawater. Tissue distribution of radioactivity was determined as well as the nature of radiolabelled metabolites in the hepatopancreas and in seawater. Fenitrooxon, 3-methyl-4-nitrophenol, desmethyl fenitrothion, desmethyl fenitrooxon, and amino fenitrothion were detected in seawater and in the hepatopancreas. In addition, 3-methyl-4-aminophenol, and the glycoside and sulfate conjugates of 3-methyl-4-aminophenol and 3-methyl-4-nitrophenol were detected in the hepatopancreas. Higher levels of fenitrooxon and lower levels of desmethyl fenitrothion were detected in the 34 ppt than in the 17 ppt seawater suggesting that metabolism influenced the toxicity at these two different salinities. There was no significant difference in the overall level of metabolites detected in the 22°C than in the 17°C seawater. The rate of uptake of fenitrothion by Callinectes also affects the toxicity as the uptake of fenitrothion was more rapid at the higher salinity as well as at the higher temperature.

INTRODUCTION

Modern agricultural and horticultural practices enhance the effects of pests and diseases and tend to propagate artificial communities which are inherently unstable and prone to pest infestation. In the natural environment, a plant which may serve as a host for one species will usually be surrounded by other less suitable plant types. This arrangement, which limits the supply of food for a particular organism along with the presence of natural predators, limits reproduction and population growth. However, large tracts of land which are densely covered by a single type of plant offer abundant food and no barriers to inhibit movement. In this artificial, yet typical agricultural environment, pest populations can build up exponentially throughout the whole crop. In an effort to prevent such occurrences, man has turned to chemical pesticides.

During the 19th century and first half of the 20th century, chemical crop protection agents were mainly inorganic substances such as derivatives of sulphur and arsenic, and metals such as lead, copper, and mercury. Derivatives of organic substances such as nicotine, pyrethrum, and coal tar distillates were sometimes employed. It was not until World War II that any substantial "advances" in the development of pesticides occurred. These advances, which stemmed from chemical warfare research, included the discovery

of DDT, organophosphate insecticides, and phenoxy herbicides (Green, 1976).

As a result of DDT's great success in World War II against body lice, typhus, and mosquitoes, it was rapidly swept into agricultural use. Due to its effectiveness against a broad spectrum of insect pests and its low cost, DDT was prone to be overused and abused. These practices, coupled with the high residual activity of DDT as well as most other organochlorine insecticides, led to the suspension by the Environmental Protection Agency (EPA) of all of its uses in January, 1973. DDT was declared to be an environmental hazard due to its accumulation in the food chain where it proved to be detrimental to certain forms of wildlife (Ware, 1978).

The chemically unstable organophosphate insecticides have virtually replaced the persistent organochlorine compounds (Gomma and Faust, 1972). Organophosphate insecticides are generally more toxic to invertebrates than are organochlorine insecticides but they are assumed to be chemically unstable or nonpersistent. Among the organophosphate insecticides, parathion (O,O-diethyl-O-p-nitrophenyl thiophosphate) is probably the most widely used. Due to its high toxicity, however, parathion has been banned in many countries, such as India. Structurally related, but much less toxic compounds such as fenitrothion [O,O-dimethyl-O-(3-methyl-4-nitrophenyl) phosphorothioate] are being used as effective substitutes for parathion (Adhya et al., 1981).

History and Use of Fenitrothion

Fenitrothion was first synthesized in 1956 by Drabek and Relikan in Czechoslovakia as a result of research aimed at developing new pesticides which were less toxic to nontarget organisms. In 1959, fenitrothion was introduced as an experimental pesticide by the Sumitomo Chemical Company in Japan (Fetts, 1975). Due to its low toxicity to mammals, it has become one of the most widely used broad spectrum insecticides (Lehotzky and Ungvary, 1976).

Fenitrothion is manufactured and sold by a variety of companies in many countries. It is marketed under trade names such as Dybar by Dupont, Fenitox by All Indian Medical Corp., Folithion by Bayer AG (Federal Republic of Germany), Novathion by Cheminova (Denmark), and Sumithion and S5660 by Sumitomo Chemical Co. Ltd. (Japan). Fenitrothion formulations are used primarily to control chewing and sucking insects on rice, orchard fruits, vegetables, cereals, cotton, and forests. Public health programs have relied on it to control flies, mosquitoes, and cockroaches (Berg, 1984). The most widely publicized uses of fenitrothion are to control the larvae of the rice stem borer, *Chilo suppressalis* (Fukunaga et al., 1969), in Japan, Spain, Korea, Formosa, China, Vietnam, Indonesia, and India, and to control the spruce bud worm, *Choristoneura fumiferana*, by aerial spraying of coniferous forests in eastern Canada. This pesticide is also used throughout Europe, East Pakistan, East Africa, United Arab Republic, Republic of China, New Zealand, and Brazil for the control of forest insect pests. Fenitrothion is not registered for use in the United States.

The suggested rates of application of fenitrothion vary for different crops and pest species to be controlled, but generally fall between the range of 0.5 and 2.0 kg active ingredient per hectare. Application at this rate is usually tolerated by most crops although injury to cotton, brassica, and orchard fruits has been occasionally observed. In 1971, over 7 million acres of Canadian forest were treated with fenitrothion. Field observations of aerial fenitrothion application have shown that 15-75% of the pesticide which was emitted from the aircraft ultimately reached the target (Krehm, 1973). In one study, fenitrothion residues were routinely detected in fields up to 3 km away from sprayed forests, and often residues were detected at distances up to 8 km from the nearest site of application (Wood, 1976). Fenitrothion concentrations of approximately 5 μg/liter have been documented in rivers following aerial spraying, and in one study the average concentration for the first 12 hours following spraying was 2.5 μg/liter (Peterson and Zitko, 1974).

Fenitrothion has a very low toxicity to mammals. The acute oral LD_{50} in rats is 800 mg/kg and the dermal LD_{50} is 1300 mg/kg. However, fenitrothion is highly toxic to insects as well as many aquatic organisms. The 48 hour LC_{50} for rainbow trout is 1.28 mg/liter, while the 3 hour LC_{50} for daphnia is 9.2 μg/liter (Miyamoto et al., 1978). Marine crustaceans are even more sensitive to fenitrothion, and organophosphates in general (Butler, 1965), as the 48 hour LC_{50} for fenitrothion in brown shrimp, Penaeus aztecus, is 2.5 μg/liter and 0.3 μg/liter for the American lobster, Homarus americanus (Butler, 1964). In general, with respect to acute toxicity, crabs and shrimp

are approximately 500 times as sensitive to organophosphate insecticides than are most fish or bivalves (Butler, 1965).

Exposure to fenitrothion at subacute levels has been shown to have undesirable behavioral effects on aquatic organisms (Hatfield and Anderson, 1972). Acetylcholine is present in the nervous tissue of aquatic crustaceans in considerable amounts (Smith, 1939). It has also been reported that autotomization, or the casting off of legs as a defensive escape mechanism in crabs, is an acetylcholine mediated reflex (Welsh and Haskin, 1939). Since the arresting of acetylcholine breakdown via inhibition of acetylcholinesterase forms the basis of action of organophosphate pesticides (Corbett, 1974), it is possible that exposure to subacute levels of fenitrothion might affect this autotomization response. This could subsequently affect survival rates.

Mode and Mechanism of Action of Fenitrothion

Studies concerning the metabolism and pharmacodynamics of fenitrothion have focused mainly on mammals and insects. These studies have demonstrated that fenitrothion is quickly absorbed from the mammalian intestinal tract as well as the insect gut. As is true for most other organophosphate insecticides, fenitrothion is relatively nontoxic and must be converted to its oxygen analog, fenitrooxon (Fig. i-1, II) [O,O-dimethyl-O-(3-methyl-4-nitrophenyl) phosphate], by mixed function oxygenases in order to exert its toxic effects. These toxic effects include inhibition of acetylcholinesterase (AChE) and cytochrome P-450 monooxygenases.

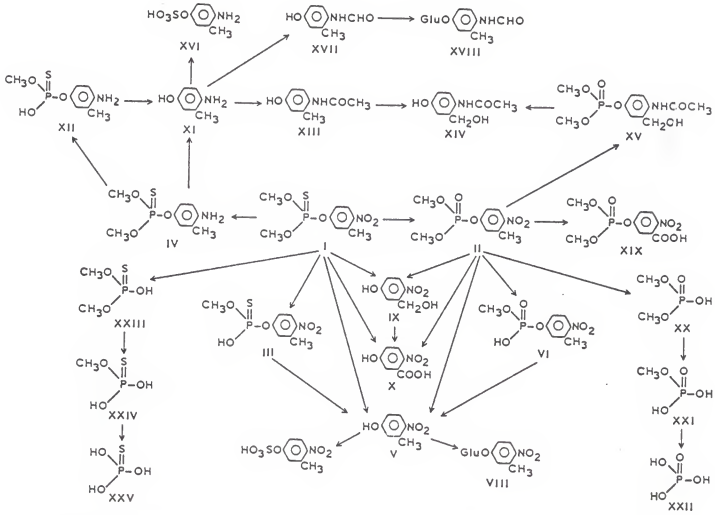

Figure 1-1. Metabolic pathways for Sumithion in mammals (Aizawa, 1982).

Inhibition of AChE is the generally accepted mode of action for organophosphate toxicity. This AChE inhibition is a competitive process between the oxon form of the organophosphate and acetylcholine (ACh), the normal substrate for AChE. The reaction between ACh and AChE yields an acetylated enzyme and free choline. The subsequent reaction of water with the acetylated enzyme results in acetic acid and the original free enzyme. When AChE is phosphorylated by fenitrooxon, a stable complex is formed, resulting in an irreversibly phosphorylated enzyme. This results in a build up of ACh at synaptic junctions which may lead to CNS related convulsions and ultimately death (Eto, 1974).

In vivo inhibition of hepatic cytochrome P-450 monooxygenases has been observed following exposure of rats to fenitrothion. Similar results with parathion have not been observed due to parathion's extreme toxicity. However, in vitro studies indicate that parathion may also inhibit cytochrome P-450 monooxygenases. These studies with parathion suggest that inhibition of cytochrome P-450 is dependent on the sulfur atom, as paraoxon, the oxygen analog of parathion, does not inhibit cytochrome P-450. It has been postulated that the events leading to the inhibition of cytochrome P-450 by organophosphates begin with the transfer of singlet oxygen from cytochrome P-450 to the organophosphate to form an S-oxide (Fig. i-2, I-IV). This S-oxide may then react internally to form a cyclic phosphorus-oxygen-sulfur intermediate (Fig. i-2, V). A cyclic shift of electrons may then result in the loss of singlet sulfur and the formation of paraoxon (VI). Generation of free sulfur may also result from a nucleophilic

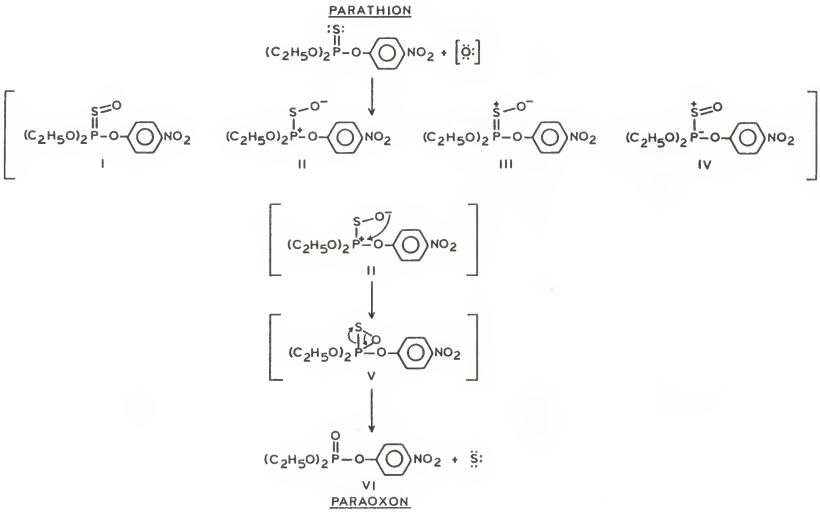

Figure i-2. Proposed chemical mechanism of the cytochrome P-450 monooxygenase catalyzed metabolism of parathion to paraoxon (Neal, 1981).

attack by water on the proposed S-oxide intermediate (Fig. i-3, II). In the case of parathion, the resulting products would be p-nitrophenol (V), diethyl phosphate (VI) and free sulfur.

If the released sulfur is in its singlet state, it would be highly reactive, and prone to bind to nucleophiles near the site of its release. In vitro studies with hepatic microsomes, purified monooxygenase systems, and labelled parathion have shown that the thionosulfur binds to the P-450 monooxygenases in the presence of NADPH. The sulfur binds to at least 4 sites on the cytochrome molecule; one of which is probably in the form of a hydro-disulfide linkage on the cysteine side chain. However, the exact nature of the binding which is responsible for the loss of cytochrome P-450 monooxygenase activity is yet to be determined (Neal, 1981).

Figure i-1 depicts the compounds which were isolated and identified in mice, rats, guinea pigs, and fish following administration of fenitrothion by various means. These pathways have been postulated solely on the identification of these compounds as metabolites. The two major routes of fenitrothion and/or fenitrooxon detoxification in mammals are dearylation and dealkylation. Dearylation results in the hydrolytic splitting of the P-O-aryl bond of fenitrothion and the subsequent formation of p-nitro-cresol (V), dimethyl phosphorothioic acid (XXIII), and dimethyl phosphoric acid (XX). Studies with parathion indicate that the oxon form is dearylated primarily by esterases resulting in phenol and dialkyl phosphate metabolites. Dealkylation results in the cleavage of methyl

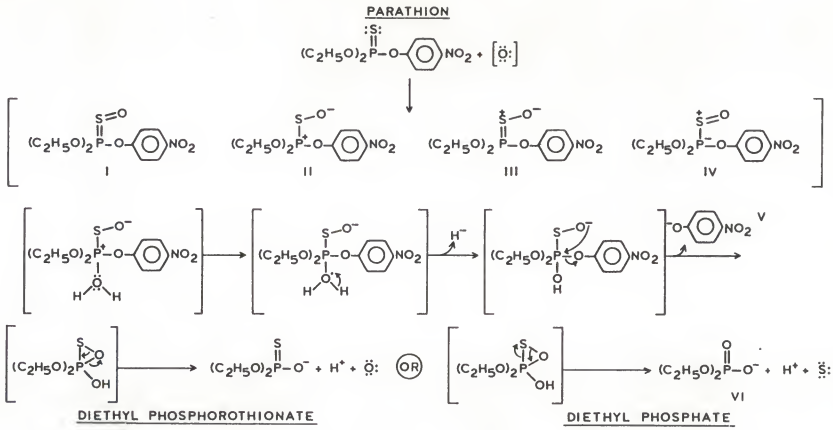

Figure i-3. Chemical mechanism for the metabolism of parathion to diethyl phosphorothionate and diethyl phosphate by the cytochrome P-450-containing monooxygenase system (Neal, 1981).

ester phosphorous bonds of fenitrothion and/or metabolites resulting in desmethyl compounds (III, VI, XII).

The degradation of fenitrothion has been investigated by in vitro studies with rats, cockroaches, and rice stem borer larvae. It was observed that while the levels of dearylation were similar in mammals and insects, rat liver homogenates demethylate fenitrothion more efficiently than did homogenates of similar organs in insects. The enzyme responsible for the formation of desmethyl products was observed predominantly in the cytosolic fraction of the liver homogenate. In similar studies with parathion, the formation of these compounds proceeded in the absence of oxygen and was inhibited by phenyl mercuric acetate. Demethylation activity was restored by the addition of reduced glutathione, suggesting that SH groups are essential to the demethylation process.

In rat liver homogenates, the addition of GSH was effective in increasing the demethylation of methyl parathion in the soluble fraction. In insect midgut and fat body homogenates, formation of desmethyl products was slight. The addition of GSH greatly increased the level of desmethyl compounds detected in the soluble fraction. Slight increases were also noted in the insoluble fraction. The addition of other SH containing compounds such as L-cystein or thioglycolic acid had little effect on the formation of desmethyl metabolites.

In vitro reaction products of liver homogenates and organophosphate insecticides were separated by TLC following a cleanup procedure. A ninhydrin positive substance with an R_f value

corresponding to that of S-methyl glutathione was hydrolyzed in HCl and rechromatographed. The hydrolysate exhibited a chromatographic pattern corresponding to glycine, glutamic acid, and S-methyl cystein. From these results it was concluded that GSH serves as a methyl group acceptor in the formation of desmethyl fenitrothion and desmethyl methyl parathion in tissue homogenates of rat liver and cockroach fat body, presumably by serving as a cofactor for glutathione-S-transferase. It was not determined if GSH participates in subsequent reactions leading to the formation of phosphate (Fukunaga et al., 1969).

Hollingsworth et al. (1967) reported that dealkylated metabolites of fenitrothion increased relative to the levels of other metabolites detected following administration of increased doses in rats. A 320-fold increase in fenitrothion dosage resulted in a 17-fold increase in the amount of dimethyl phosphoric acid detected in urine. Simultaneously, a 620-fold increase in the amount of desmethyl fenitrothion was detected. On the basis of these observations, they suggested that dearylation, which predominated following lower levels of fenitrothion administration, becomes less important as saturation of the associated enzymes occur.

Much less literature exists concerning the dearylation of fenitrothion. This may be due to the fact that comparable levels of dearylation have been observed in both mammals and insects and thus it seems likely that these reactions are not responsible for the large difference in mammalian and insect toxicity to fenitrothion (Nakatsugawa and Dahm, 1967).

Amino fenitrothion and desmethyl amino fenitrothion have been identified as fenitrothion metabolites in soil (Adhya et al., 1981). One study also reported the occurrence of these reduced compounds in the urine of mice following exposure to fenitrothion (Miyamato et al., 1976).

Miyamato et al. (1976) surmised that fenitrothion's aromatic methyl group may be oxidized to an alcohol or carboxyllic acid as indicated by the presence of four metabolites (Fig. i-1, X, IX, XIV, XV) including 5-hydroxy-2-nitro benzyl alcohol and 5-hydroxy-2-nitro benzoic acid as urinary metabolites of fenitrothion in orally treated rats. They also documented sulfate (XII, XIV) and glucuronic acid (VIII, XVIII) conjugated metabolites of fenitrothion in rats, rabbits, and dogs. Evidence by these researchers indicate the conversions of the aromatic nitro group to acetyl amino phenyl (XIII-XV) and formyl amino phenyl (XVII, XVIII) groups during the metabolism fenitrothion in mammals.

Distribution and Life Cycle of CALLINECTES SAPIDUS

Many marine crustaceans such as shrimp, lobsters, and crabs as well as other economically important species spend at least part of their lives in estuaries. The estuarine environment is a mixing zone between our fresh water drainage basins and the ocean, and constitutes the most important area for the production of marine crops. This zone can make maximum utilization of sunlight energy and nutrients derived from the land in providing animal food. Unfortunately, organic materials, including pesticides, are carried down stream and tend to

accumulate in estuaries, posing a serious threat to this environment (Nybakken, 1982).

In order to estimate the possibility of exposure of marine crustaceans such as the blue crab (Callinectes sapidus) to pesticide runoff, one needs to consider their life cycle. Blue crabs are common along the entire Atlantic and Gulf coasts of the United States, and may be found from Nova Scotia to northern Argentina. They also inhabit coastal waters of southwest France, Denmark, and the Netherlands as well as the Adriatic, eastern Mediterranean and Black Seas. Blue crabs are primarily found in water up to 35 meters in depth. This species can also tolerate a wide range in salinity as they have been taken from fresh water springs in Florida as well as hypersaline lagoons in Mexico. Blue crabs are omnivores, and their usual diet may consist of a variety of fish, invertebrates and plant material.

Blue crabs reach the adult stage approximately 12 to 18 months after hatching, and typically live from 2 to 3 years. Mating takes place in the low salinity water of estuaries. Females mate only once, at the transition of their instar molt into the adult, whereas males may mate more than once. One to two months later, spawning occurs in higher salinity waters at the mouths of estuaries and as far as 5 to 6 km offshore. At the time of spawning, which usually occurs from spring to early fall in the eastern U.S., the female lays from 700,000 to 2 million eggs. She carries these eggs until they hatch 7 to 14 days later. While initial larval development takes place offshore, there is a shoreward movement toward the estuarine nursery ground

during the first few crab stages. Blue crabs then spend the majority of their lives in estuaries. The males generally remain in one estuarine area, while the females show the offshore/onshore migrational pattern related to their breeding cycle (Oesterling, 1976).

The Dissertation Problem

Upon considering *Callinectes*' life cycle (Oesterling, 1976), the toxicity of organophosphate insecticides to marine crustaceans (Butler, 1965, 1964), and the reported levels of organophosphate insecticides in rivers following aerial spraying (Peterson and Zitko, 1974), it seemed likely that *Callinectes* could be exposed to harmful levels of these insecticides. By disturbing the ecological balance of the river, estuary, and coastal environment, organophosphate insecticide contamination could adversely affect crab populations for future generations by destroying the breeding stock. Also, it is possible that crabs may metabolize organophosphate insecticides to compounds which may be harmful to consumers or other organisms in the marine environment.

The purpose of this research was to estimate the possible hazards associated with organophosphate insecticide contamination of coastal and estuarine areas by investigating the toxicity and metabolism of the insecticide fenitrothion (Fig. i-1, I) to *Callinectes sapidus*. *In vivo* studies were utilized in chapter I to determine the effects of salinity, temperature, and a simulated tidal cycle on the acute toxicity of fenitrothion to *C. sapidus*. The effects of exposure of

C. sapidus to sublacutely toxic levels of fenitrothion on the autotomization response were also determined. The *in vitro* experiments summarized in chapter II correlate differences in the rates of metabolism of fenitrothion by *C. sapidus*, which had been acclimated to various salinities and temperature, to the toxicity differences reported in chapter I. *In vitro* studies were also employed to clarify metabolic pathways. Tissue distribution of cytochrome P-450 monooxygenase and glutathione-S-transferase activities, and the effects of the addition of a cytochrome P-450 monooxygenase inhibitor and various levels of reduced glutathione on the *in vitro* metabolism of fenitrothion are also reported in chapter II. Chapter III reports on the synthesis and utilization of ^{14}C-labelled fenitrothion to determine the effects of salinity and temperature on the uptake and *in vivo* metabolism of fenitrothion by *C. sapidus*.

CHAPTER I
THE EFFECTS OF TEMPERATURE, SALINITY, AND A SIMULATED TIDAL CYCLE ON THE TOXICITY OF FENITROTHION TO *CALLINECTES SAPIDUS*

Materials and Methods

Animals

Approximately 400 intermolt blue crabs, *Callinectes sapidus*, with a carapace width of 8.5-11.0 cm were used. The crabs were either captured with dipnets in the littoral zone off the south shore of Sea Horse Key, Florida, or obtained from a commercial crabbing operation in Cedar Key, Florida. The crabs' claws were banded to prevent cannibalism and they were then packed in ice and seaweed for transport to Gainesville. Upon arrival at our laboratory, they were immediately placed in holding tanks containing 22 ppt seawater, approximately the salinity from which the crabs were harvested. After 48 hours, the crabs were then transferred to aquaria containing either 17 or 34 ppt seawater at 17 or 22°C in order to allow them to acclimate for 14 days prior to use. All crabs were fed approximately 4 g of squid on alternate days during this acclimation period. Feeding was terminated 48 hr prior to all toxicity tests.

Test Chambers and Experimental Conditions

Glass aquaria measuring 75 x 32 x 36 cm (L x W x H) were used for all acclimation and experimental procedures. These aquaria were housed in Scherer Model CEL 51237 environmental chambers. Seawater

was obtained from the C.V. Whitney Laboratory, Marineland, Florida, and diluted to the desired salinity, if necessary, with deionized water. Salinity measurements were determined with a refractometer (American Optical Corp., Model 10423). All aquaria were aerated by passing a fine stream of air through glass disposable pipettes. A 16 hr light and 8 hr dark photo period was used during all toxicity tests and acclimation periods. Unless otherwise noted, toxicity tests were performed at 22±1°C.

Toxicant

All reagents used in this experiment were of reagent or analytical grade. Fenitrothion was prepared by the method of Hollingsworth (1966) and 5-6 g purified by silica gel chromatography on a 50 x 4 cm column with acetone:hexane (90:10) as the eluting solvent. The solvent was removed from appropriate combined fractions and the resulting fenitrothion was analyzed for purity by HPLC using a Waters C_{18} μBondpack column, model 6000A solvent delivery system, model 440 absorbance detector at 280 nm, and a 50:50 (methanol:water) mobile phase at a flow rate of 1.5 ml/min. Pure samples were quantified gravimetrically and stock solutions (280 mg/ml) were prepared in ethanol.

Fenitrothion Extraction and Analysis

Fenitrothion concentrations in seawater were monitored by modifying the procedure of Lyle (1975). Duplicate 1 liter aliquots of seawater were removed from the test aquaria at the beginning of each experiment and at 36 hr intervals thereafter. This seawater was then eluted through a separatory funnel containing 100 ml wet volume of

XAD-4 resin (Applied Science, Deerfield, IL) slurried in methanol at a flow rate of approximately 1 liter/hr. The XAD-4 resin was eluted with 300 ml acetone to recover the fenitrothion. The volume of this eluant was reduced by low pressure rotary evaporation until only the aqueous phase remained. This aqueous phase was extracted three times using 60 ml of ethyl ether:ethyl acetate (95:5) each time and the organic extracts pooled and reduced to approximately 5 ml by rotary evaporation *in vacuo*. This concentrate was transferred to a graduated centrifuge tube and reduced to 1.0 ml with a stream of N_2 at room temperature. The fenitrothion levels in seawater were then quantified by analyzing 20 µl of this extract with the previously described HPLC system and a 45:55 (methanol:water) mobile phase. Recovery of fenitrothion from spiked seawater samples was 96.7±1.14%.

Testing

Crabs were permitted to acclimate to test salinity and temperature conditions for 2 weeks prior to all toxicity tests. The LC_{50} for *Callinectes* exposed to fenitrothion was determined by following the suggested procedures for static toxicity tests in *Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians* (Committee on Methods for Toxicity Tests with Aquatic Organisms, 1975) except that the recommended loading of organisms for a flow through toxicity test was used. Acceptable dissolved oxygen concentrations were maintained by aeration of the test chambers. The LC_{50} experiment was performed with 5 or 6 crabs per aquarium. The approximate size distribution and sex ratio of 5 males to 1 female

in each aquarium was kept constant. Two replicates were performed at each fenitrothion concentration at 22°C.

To determine the effects of salinity and temperature on the acute toxicity of fenitrothion to Callinectes, 192 crabs were divided into groups of 12 crabs, each group containing approximately the same size distribution and ratio of males to females. Groups were then randomly assigned to test aquaria containing 70 liters of seawater. Experimental conditions for each aquarium were one of the four following salinity and temperature combinations: 17 ppt 17°C, 17 ppt 22°C, 34 ppt 17°C, 34 ppt 22°C. Aliquots containing 1400 µg fenitrothion in 50 µl ethanol were added to each tank to give an initial concentration of 20 µg/liter. Fenitrothion levels in the seawater were determined at 0, 36 and 72 hr. Control tanks received 50 µl ethanol instead of the fenitrothion:ethanol stock solution. At each of the four temperature:salinity combinations, 36 crabs were exposed to fenitrothion and 12 crabs were used as a control. The crabs were observed at 2 hr intervals at which time they were gently prodded with a glass rod. Dead crabs, those which showed no response to prodding, were removed immediately and time of death was recorded. After 72 hr the experiment was terminated, and all living crabs were assigned a value of 72 hr. Mean death times were then calculated for each experimental group.

In order to investigate whether stress due to salinity changes affects the acute toxicity of fenitrothion to Callinectes, another batch of crabs was divided into 8 groups of 14, each with the 11:3 ratio of male to female and approximately the same size

distribution. Two groups were randomly assigned to aquaria containing 70 liters of either 17 ppt or 34 ppt seawater and fenitrothion at 14 µg/liter. To simulate concurrent exposure to organophosphate pesticides and tidal cycles, 2 other groups were transferred between 2 aquaria every 6 hr. One of these aquaria contained 17 ppt seawater while the other contained 34 ppt seawater. Both also contained fenitrothion at 14 µg/liter. Control groups were treated identically except that these crabs were exposed only to the carrier solvent. All crabs were observed at 2 hr intervals, and a mean death time for each group was determined as previously stated.

A procedure developed by Welsh and Haskin (1939) was slightly modified to determine the effects of subacute exposure on Callinectes' ability to autotomize. Following the 2 week acclimation period, Callinectes were divided into 5 groups of 10, each group containing 7 males and 3 females. The groups were randomly assigned to aerated aquaria containing 70 liters of 34 ppt seawater at 22°C and initial fenitrothion concentrations of 0, 0.01, 0.1, 1.0 or 5.0 µg/liter. After 72 hr exposure to fenitrothion at these various subacute levels, the merus on every limb of each Callinectes was crushed with a pair of needle-nose pliers by holding them closed for 2 sec and then releasing. The limbs were crushed in a random order and the total number of limbs autotomized was recorded so that a mean number of limbs autotomized per crab could be determined for each group. No distinction was made between walking legs, chela and swimming appendages.

Statistical Methods

Acute lethality test data were analyzed by the probit analysis method of Finney (1971) to determine the concentration of fenitrothion to kill 50% of the test animals within 96 hr and the 95% confidence intervals. The effects of salinity and temperature on acute toxicity were determined by analysis of variance on the mean death time of each experimental group using the SAS General Linear Model procedure. The results of the tidal cycle stress effects on toxicity experiments were analyzed by the SAS analysis of variance procedure (SAS Institute, 1983). The Student's t-test was utilized to compare the group means in the autotomization experiment (Mendenhall, 1975).

Results

Fenitrothion levels in seawater decreased by approximately 28% every 24 hr during the toxicity tests. Unless otherwise stated, all reported fenitrothion concentrations are mean concentrations determined during that particular experiment. The 96 hr LC_{50} for fenitrothion exposed *Callinectes sapidus* in 34 ppt seawater at 22°C was found to be 8.6 µg/liter with a 95% confidence interval of 7.4-9.9 µg/liter. The Chi Square for the LC_{50} calculation was 0.25 and the equation for the toxicity curve was 5.63 x -1.05.

The results of the experiment designed to investigate the effects of salinity and temperature on toxicity are presented in Table 1-1. Analysis of these data by the SAS General Linear Model procedure indicates that there was not a significant interaction of salinity and temperature effects on acute toxicity as indicated by mean death time

Table 1-1. The effect of salinity and temperature on mean death time of *Callinectes* exposed to fenitrothion

Salt (ppt)	Temperature (°C)	N	Mean death time (hr)
17	17	36	61.3
17	22	36	57.6
34	17	24	55.8
34	22	36	43.5

(P=0.2040). The independent effects of salinity and temperature on acute toxicity were highly significant as indicated by the significance of the F statistics for these parameters (P=0.0042 and P=0.0186, respectively). The least square mean death time for 17 ppt salinity was 59.5 hr while the comparable value for 34 ppt was 49.7 hr (Table 1-2). This difference, which is highly significant (P=0.0042),

Table 1-2. Least square means for the independent effects of salinity and temperature on mean death time of *Callinectes sapidus* exposed to fenitrothion

Variables	Least square mean death time (hr)	Prob > |T| Ho: LS Mean 1 = LS Mean 2
Salt		
17 ppt	59.5	0.0042
34 ppt	49.7	
Temperature		
17°C	58.6	0.0186
22°C	50.6	

indicates that fenitrothion is more toxic to Callinectes at salinities of 34 ppt than at 17 ppt. The results of the analysis as presented in Table 1-2 also indicate that the difference between the least square mean death time for Callinectes exposed to fenitrothion at 17°C which was 58.6 hr and at 22°C which was 50.6 hr is also highly significant (P=0.0186). This indicates the fenitrothion was more toxic at the higher temperature. When compared by the Duncan's Multiple Range Test (data not shown) the mean death time for the 34 ppt 22°C group (Table 1-1), the combination of highest temperature and highest salinity, was significantly less than that of the other groups (P=0.05). The mean death time for those control groups were not significantly different from 72 hr as one crab died in each control group during the experiment.

The Duncan's Multiple Range Test on mean death time data from Callinectes which were simultaneously exposed to fenitrothion and simulated tidal cycle (Table 1-3) indicate that the mean death times for the two static salinity and one variable salinity control groups were not significantly different from each other. The mean death times for all of the pesticide exposed groups were significantly different from all the controls. Furthermore, the mean death time for the crabs exposed to fenitrothion in conjunction with the simulated tidal cycle of salinity changes every 6 hr was significantly less than that observed for the two groups exposed to the pesticide at static salinities.

The effects of exposure to subacute levels of fenitrothion on autotomization were analyzed by a Student's t-test (Mendenhall, 1975)

Table 1-3. Duncan's Multiple Range Test on the effect of a simulated tidal cycle on fenitrothion toxicity to Callinectes sapidus

Fenitrothion (µg/l)	Salinity (ppt)	Mean time to death (hr)	Duncan grouping[a]
Controls			
0	34	71.5	A
0	17	71.1	A
0	17/34[b]	66.6	A B
Experimental			
14	17	57.1	C B
14	34	52.1	C
14	17/34[b]	39.6	D

[a] Means with the same letter are not significantly different α=0.05 with 78 df.

[b] 17/34 = simulated tidal cycle.

Note: Each group consists of 14 observations.

and are shown in Fig. 1-1. None of these crabs died during the 72 hr exposure to fenitrothion at any of the subacute levels. The mean number of limbs autotomized by Callinectes following exposure was significantly different from the controls at initial concentrations of 5.0, 1.0 and 0.1 µg/liter ($P<0.01$). The mean number of limbs autotomized by Callinectes following exposure to 0.01 µg/liter fenitrothion was not significantly different from the control ($P<0.05$).

Discussion

The 96 hr LC_{50} of 8.6 (95% C.I. = 7.4-9.9) µg/liter for Callinectes sapidus exposed to fenitrothion is quite similar to LC_{50}

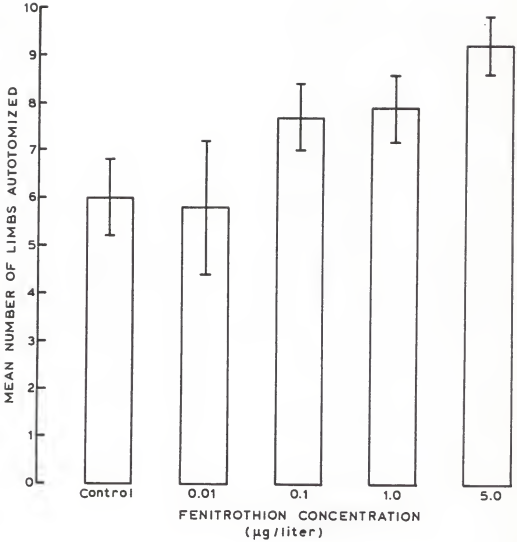

Figure 1-1. The effect of subacute exposure to fenitrothion on autotomization by Callinectes sapidus.

of 11 (5-21) μg/liter that was reported for the aquatic invertebrate Daphnia pulex (Johnson and Finley, 1980). The LC_{50} values for the amphipod Gammacus fasciatus and for the stonefly (Pteronarcys californica) are 3 and 4 (3-6) μg/liter, respectively (Johnson and Finley, 1980). Following exposure to fenitrothion, the 96 hr LC_{50} values for 17 species of freshwater fish ranged from 1.7 to 5.0 mg/liter (Johnson and Finley, 1980). The 96 hr LC_{50} for small and large crayfish is 10 and 30 μg/liter, respectively (McLeese, 1976). The only other fenitrothion toxicity data for marine crustacea which could be found in the literature were the 48 hr LC_{50} value of 2.5 μg/liter for the shrimp, Penaeus aztecus, and 1.0 μg/liter for the American lobster, Homarus americanus (McLeese, 1976). Comparison of these data indicates that fenitrothion is less toxic to blue crabs than to shrimp and lobster. These data also indicate that aquatic invertebrates are three orders of magnitude more sensitive to fenitrothion than are most aquatic vertebrates.

The reported maximum concentration of fenitrothion in fresh water streams following spraying rarely exceeds 15 μg/liter (Eidt and Sundaran, 1975) with an average peak of about 5 μg/liter. In one study, fenitrothion concentrations of 20 μg/liter were found in a Canadian river (Mallet and Volpe, 1982). Fenitrothion concentrations usually declined to about 50% by 6 hr and to 10% by 15 hr post application (McLeese, 1976). These data suggest that current application practices result in fenitrothion levels in streams which are of the same order of magnitude as those levels which would cause acute toxicity to Callinectes sapidus in 34 ppt salinity seawater at

22°C. Due to the latitudinal onshore/offshore migrational movements of female Callinectes and the limited movement of males, Callinectes do not usually move from one estuarine system to adjacent estuarine systems (Oesterling, 1976). Thus a reduction in the crab population in a particular estuary resulting from agricultural run-off would not be readily replenished by crabs from nearby estuaries. It is possible that one incidence of organophosphate contamination could affect the ecology of an estuary for many years.

The toxicity data presented in Tables 1-1 and 1-2 indicate that increased toxicity is associated with increased salinity or increased temperature. It is unlikely that this increase in toxicity is directly attributable to respiration rates or rates of ventilation. It has been shown that Callinectes' respiration rate decreases as salinity increases (King, 1965) which contrasts the increased rate of respiration associated with an increase of temperature in marine invertebrates (Kinne, 1970). The possibility that the difference in toxicity observed was due to differences in the ratio of toxification and detoxification enzyme activity at different temperature and intracellular osmolarity is reported in chapters II and III.

Subacute exposure of aquatic organisms to fenitrothion at levels of 1.0 mg/liter for 24 hr has been shown to inhibit the learning ability of Atlantic parr salmon (Hatfield and Johansen, 1972) and to increase the susceptibility of parr to predation by brook trout (Hatfield and Anderson, 1972). Yasano et al. (1980) have also demonstrated that subacute fenitrothion exposure affected reproduction in the guppy. Conclusions should be drawn cautiously from the results

of the autotomization experiment (Fig. 1-1) as the consistency of force applied to the hand-held pliers could have been somewhat variable. However, the results indicate that subacute exposure of Callinectes to fenitrothion increased the probability that a limb will autotomize in response to a given stimulus. This effect might be due to a decrease in acetylcholinesterase activity. This could result in an increased localized concentration of acetylcholine which in turn would increase the likelihood of an acetylcholine mediated autotomization response. This increased autotomization response was observed to be significant at levels as low as 0.1 µg/liter which is more than an order of magnitude less than fenitrothion concentrations detected in streams 15 hr after aerial application (Eidt and Sundaran, 1975). Autotomized limbs are regenerated only following a molt, but two successive molts are required for the limb to attain its normal size. While the loss of a limb stimulates the onset of the next molt, a less than normal size increase is associated with this molt since resources are diverted to the regenerating limb. It is likely that this increase in autotomization observed for fenitrothion exposed crabs could have a drastic affect on adults since the time between successive molts increases as the crab matures. It has also been shown that chronic exposure to organophosphate insecticides inhibits molting in crabs (Reddy et al., 1985). Furthermore, in many species mature crabs do not molt after attaining an upper limit in size, and an autotomized limb would never be regenerated (Warner, 1977; Waterman, 1960). This could decrease an adult's survival rate by inhibiting its ability to procure food and defend itself.

The data in Table 1-3 indicate that exposure of crabs to the simulated tidal cycle regime of a 17 ppt change in salinity every 6 hr increased the acute toxicity of fenitrothion as compared to crabs which had been exposed to the same pesticide concentration at static salinities. Since the mean death times for all three control groups are not significantly different from each other, and since the mean death time for the group which was exposed to fenitrothion during the tidal cycle exposure is significantly different from the static salinity fenitrothion exposed group, it appears that there is an increased toxicity associated with the simultaneous exposure to tidal cycles and fenitrothion. It is possible that crabs may migrate during a tidal cycle in order to reduce the magnitude of salinity changes to which they are exposed, and that the salinity changes associated with normal tidal cycles may be more gradual than used in this model.

Thus, with respect to acute toxicity, these data suggest that the reported levels of fenitrothion in streams approach those which would cause an increased mortality to Callinectes at static salinities. Since Callinectes are less susceptible to the toxic effects of fenitrothion than other marine crustacea such as shrimp and lobsters, it is likely that these other economically valuable marine crustacea would be even more vulnerable to the toxic effects of organophosphate pesticides. Subacute effects which are more difficult to quantitate but which also could affect Callinectes survival rate are quite likely to occur due to pesticide runoff following normal pesticide application procedures. These data also indicate that fenitrothion toxicity increased with increasing salinity, increasing temperature

and with the stress which accompanied exposure to a simulated tidal cycle. Furthermore, the currently acceptable practice of performing toxicity tests at static salinities may underestimate the toxic effects of organophosphate pesticides in estuarine environments.

CHAPTER II
THE EFFECTS OF TEMPERATURE AND SALINITY ON THE *IN VITRO* METABOLISM OF FENITROTHION BY *CALLINECTES SAPIDUS*

Materials and Methods

Animals

Male *Callinectes* were obtained, transported and acclimated for 2 weeks to 17°C 17 ppt (parts per thousand), 22°C 17 ppt, or 17°C 34 ppt seawater as described previously.

Chemicals

Fenitrothion [O,O-dimethyl-O-(3-methyl-4-nitrophenyl) phosporothioate] (Fig. i-1, I) was synthesized by the condensation of dimethyl phosphorochloridothionate and sodium 3-methyl-4-nitrophenolate (Hollingsworth et al., 1967). The IR and UV spectra of this material were identical to published spectra. The refractive index was n_D^{25} 1.5531 (lit n_D^{25} 1.5528) (Nishizawa et al., 1961). HPLC and GC retention times of the synthesized material were identical to that of a purchased fenitrothion standard (Chem Service Inc., Westchester, PA). Mass spectral analysis of the synthesized material gave the same major peaks as published spectra (Hallet et al., 1974). A parent peak at 277 m/e was detected. Peaks were also detected at 152, 125, 109, 93, and 79 m/e with the relative amounts of these fragments being 3, 100, 61, 28, and 41%.

Fenitrooxon [O,O-dimethyl-O-(3-methyl-4-nitrophenyl) phosphate] (Fig. i-1, II) was synthesized by the condensation reaction between dimethyl phosphorochloridate and sodium 2-methyl-4-nitrophenolate (Hollingsworth, 1966). The synthesized fenitrooxon had a boiling point of 147°C (0.7 mm) and refractive index of n_D^{25} 1.5160 as compared to literature values of 147-147°C (0.7 mm) and n_D^{25} 1.5147 (Nishizawa et al., 1961). The mass spectrum was in agreement with the published spectrum (Hallet et al., 1974). Fenitrooxon showed a parent peak at 261 m/e with additional peaks at 244, 127, 109, 79, and 63. The relative amounts of these fragments were 44, 12, 100, 21, and 11%.

S-Methyl fenitrothion [O,S-dimethyl-O-(3-methyl-4-nitrophenyl) phosphate] was prepared by thermal isomerization of fenitrothion at 165°C for 30 minutes. Mass spectrum of the product was in agreement with the published spectrum (Hallet et al., 1974) as the parent peak was noted at 277 m/e. Other peaks were detected at 260, 153, 136, 125, 77, 47, and 40 at relative amounts of 29, 6, 12, 100, 9, 15, and 14%.

Amino fenitrothion [O,O-dimethyl-O-(3-methyl-4-amino phenyl) phosphorothioate] (Fig. i-1, IV) was prepared by the reduction of fenitrothion in the presence of HCl and tin, and confirmation was made by mass spectral analysis (Hallet et al., 1974). A parent peak was observed at 247 m/e. Other peaks were found at 150, 138, 125, 122, 106, 93, and 77 m/e at relative amounts of 100, 23, 58, 13, 26, and 27%.

Desmethyl fenitrothion was prepared by the action of thiophenol on fenitrothion as described by Hollingsworth (1966). The product

exhibited TLC retention times relative to fenitrothion which were in agreement with the literature (Miyamoto et al., 1976). Purity was established following visualization by spraying the plates with sulfuric acid and subsequent heating. Conversion of desmethyl fenitrothion to fenitrothion by treatment with diazomethane was also accomplished and confirmed by mass spectrometry.

The purity of all synthesized chemicals was established by GC-FID on a Hewlett Packard 5840 gas chromatograph equipped with a 1.8 meters x 2 mm column packed with 4% SE 30/6% QF1 on 80-100 mesh chromsorb W (Applied Science, Deerfield, IL). Column temperature was programmed from 70°C to 250°C at 5°C per minute. N_2 was used as a carrier gas at a flow rate of 40 ml/min with the injection port and detector at 200°C. All of the synthesized chemical standards were at least 95% pure.

The compound 3-methyl-4-nitrophenol was obtained from Sigma Chemical Co. and purified by silica gel chromatography. All reagents used in this experiment were of analytical grade or better. All solvents were obtained from Fisher Scientific and were of reagent grade. HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid, glucose-6 phosphate, glucose-6-phosphate dehydrogenase (Type XV) and NADP were purchased from Sigma Chemical Co. SKF-525A (1-diethylaminoethyl 2,2-diphenylvalerate) was a gift from Dr. Allen Neims, Department of Pharmacology, University of Florida. All other chemicals were purchased from Aldrich Chemical Co.

In Vitro Metabolism

The hepatopancreas was removed from weighed, sexually mature, male Callinectes, then weighed, and placed in ice-cold buffer (1.15% KCl, 0.02 M HEPES pH 7.4). It was then homogenized using 4 passes of a motor driven Teflon/glass tissue grinder and fractionated into cytosolic and microsomal fractions on a Sorvall OTD-2 ultra centrifuge (Conner and Singer, 1981). Protein concentrations of the fractions were determined with Coomassie Blue (Bradford, 1976). Cytosolic and microsomal fractions were adjusted to 5 mg/ml or 12.5 mg/ml, respectively, using the KCl/HEPES buffer. A Beckman DU-8 spectrophotometer was used to determine the cytochrome P-450 content of the microsomal fractions from carbon monoxide difference spectra of dithionite reduced microsomal suspensions (Omura and Sato, 1964).

The metabolism of fenitrothion by the cytosolic fraction was investigated by adding either 2 ml of cytosolic protein suspension containing 10 mg protein or 2 ml of buffer for the controls to a test tube (15 ml) containing 1 ml of HEPES and 1 ml reduced glutathione (GSH) solution to give final concentrations of 0.1 M and 10 mM, respectively. The tubes were placed in a shaking, temperature controlled water bath, and after a 2 minute temperature equilibration period, 500 µg fenitrothion in 90 µl ethanol was added. The effect of increased GSH concentration was determined by varying the amount of GSH added to the cytosolic incubate. All metabolism studies were done at the temperature and salinity to which the crabs had been acclimated. Metabolism was allowed to proceed for 6 minutes at 22°C or 12 minutes at 17°C since previous studies indicated that the

formation of all detectable metabolites was first order for at least this duration at these temperatures. At the end of this incubation period, 2 ml of acetone were added to each tube and the tubes were placed in an ice cold water bath.

Microsomal metabolism of fenitrothion and fenitrooxon was determined by adding 500 µg of fenitrothion or fenitrooxon in 90 µl of ethanol to a test tube (15 ml) containing 20 µM $MgSO_4$, 400 µM pH 7.4 HEPES, a NADPH generating system consistng of 8 µM $NADP^+$, 30 µM glucose-6-phosphate and 4 units glucose-6-phosphate dehydrogenase along with 20-30 mg microsomal protein in a final volume of 4 ml. All other incubation parameters were identical to those used to determine cytosolic metabolism. The effects of SKF-525A on microsomal metabolism were investigated by adding 50 µg SKF-525A to this microsomal incubate.

Identification and Quantification of In Vitro Metabolites

The incubates were extracted three times with 5 ml ethyl acetate. The aqueous phase was saved and the organic extracts were pooled, dried with Na_2SO_4 and evaporated to dryness under a gentle stream of N_2 at 35°C. The resulting residue was dissolved in 500 µl ethyl acetate and analyzed for the metabolites in Table 2-1 using a Waters C_{18} µBondpack column, model 440 absorbance detector set at a wavelength of 280 nanometers, and a model 6000A solvent delivery system at a flow rate of 1.5 ml of 45% methanol per minute. Aliquots of this concentrate were also analyzed by GC-FPD using the equipment and conditions cited earlier. Metabolites were identified and quantified by comparing retention times and peak heights respectively

Table 2-1. Retention times of fenitrothion and its metabolites by HPLC and GC

Compound	Retention time in minutes[a]	
	HPLC[b]	GC[c]
Fenitrothion	44.2	11.5
Fenitrooxon	10.0	13.6
S-Methyl Fenitrothion	11.7	21.4
Amino Fenitrothion	9.1	8.5
3-Methyl-4-nitrophenol	5.8	--

[a] Mean times of at least three replicate injections.

[b] Waters C_{18} μBondpack column, mobile phase = 45% methanol, flow = 1.5 ml/min absorbance = 280 nanometers.

[c] 1.8 meters x 2 mm i.d. column packed with 4% SE 30/6% QF1 on 80-100 mesh chromsorb W, oven temp = 194°C, carrier gas = N_2 at 40 ml/min.

with those of the standards listed in Table 2-1. Metabolite identification was confirmed by GC/mass spectrometry. Mass spectra were generated on a Hewlett-Packard 5985 GC/MS system operated in the electron impact ionization mode (70eV; source temperature 200°C). Samples (2 μl) were injected in the splitless mode onto a 30 meter x 0.25 mm DB-5 capillary column (J&W Scientific, Inc.) which was interfaced directly into the MS source. After a 2 minute hold at 50°C, the column temperature programmed to 280°C at 5°C per minute. The mass spectrometer was scanned from m/z 45 to 450 every 0.4 second and spectra were collected for 50 minutes. Spectra were then compared to those of authentic standards.

The pH of the aqueous phase was adjusted to 1.0 and extracted three times with 5 ml ethyl acetate. Extracts were pooled, dried with Na_2SO_4 and reduced to approximately 1 ml under a gentle stream of N_2 at 35°C. In order to methylate any desmethyl metabolites, the extracts were allowed to stand at room temperature for 20 minutes following the addition of 2 ml ethereal diazomethane solution (Forbes et al., 1975). This reaction mixture was reduced to dryness under a stream of N_2 at 35°C and redissolved in 500 µl ethyl acetate. Aliquots of this extract were then analyzed by GC-FPD and GC/MS.

Tissue Distribution of Glutathione-S-Transferase Activity and Cytochrome P-450 Monooxygenase Activity

The pyloric stomach, gills, heart, testis and hepatopancreas were removed from sexually mature male *Callinectes* which had been acclimated to 34 ppt 22°C seawater. Muscle was removed from the chelipeds and hemolymph was drawn from beneath the soft tissue of the cheliped between the merus and the carpus. Tissue homogenates were prepared (Bihari et al., 1984) and fractionated into cytosolic and washed microsomal fractions as described earlier (Conner and Singer, 1981). Aniline hydroxylase activity was used as a measure of cytochrome P-450 monooxygenase activity as reported by Pohl et al. (1974) and GSH transferase activity was monitored as reported by James et al. (1979a) using 1-chloro-2,4-dinitrobenzene as a substrate.

Results

The data presented in Tables 2-2 and 2-3 show that 3-methyl-4-nitrophenol and fenitrooxon were enzymatically formed in the microsomal incubate while desmethyl fenitrothion was formed from

Table 2-2. The effects of acclimation salinity on the *in vitro* metabolism of fenitrothion by *Callinectes sapidus* hepatopancreas[a]

Metabolite	nmol formed min^{-1} ±95% confidence limit				
	Microsomal		Cytosolic		Control (Nonenzymatic)
	17 ppt	34 ppt	17 ppt	34 ppt	
3-methyl-4-nitrophenol	245.0 ± 17.0	180.0 ± 16.3	ND	ND	6.2 ± 2.43
fenitrooxon	0.8 ± 0.1	1.5 ± 0.4	ND	ND	ND
desmethyl fenitrooxon	ND	ND	46.0 ± 10.0	17.0 ± 1.0	0.96 ± 0.25
S-methyl fenitrothion	ND	ND	ND	ND	0.63 ± 0.30

[a] Data obtained from 3 replicate experiments run at 22°C. The hepatopancreases from 5-6 crabs were combined to prepare subcellular fractions for each experiment. Microsomal and cytosolic incubates contained 25 or 10 mg protein, respectively. Values for microsomal and cytosolic metabolism are corrected for nonenzymatic product formation, and at least 94 ± 3% of parent compound was accounted for in all experiments.

ND = Values were not significantly different from control values.

Table 2-3. The effects of acclimation temperature on the *in vitro* metabolism of fenitrothion by *Callinectes sapidus* hepatopancreas[a]

Metabolite	nmol formed min^{-1} ±95% confidence limit					
	Microsomal		Cytosolic		Control (Nonenzymatic)	
	17°C	22°C	17°C	22°C	17°C	22°C
3-methyl-4-nitrophenol	35.0 ± 1.5	85.0 ± 8.8	ND	ND	4.2 ± 1.3	6.75 ± 1.4
fenitrooxon	1.0 ± 0.2	5.3 ± 4.0	ND	ND	ND	ND
desmethyl fenitrooxon	ND	ND	4.5 ± 0.3	30.0 ± 6.1	0.61 ± 0.15	1.0 ± 0.2
S-methyl fenitrothion	ND	ND	ND	ND	0.56 ± 0.02	0.75 ± 0.05

[a] Data obtained from 3 replicate experiments using crabs which had been acclimated to 34 ppt seawater at either 17°C or 22°C. The hepatopancreases from 5-6 crabs were combined to prepare subcellular fractions for each experiment. Microsomal and cytosolic incubates contained 25 or 10 mg protein, respectively. Values for microsomal and cytosolic metabolism are corrected for nonenzymatic product formation and at least 90 ± 6% of parent compound was accounted for in all experiments.

ND = Values were not significantly different from control values.

fenitrothion in the cytosolic incubate. Nonenzymatic formation of 3-methyl-4-nitrophenol, desmethyl fenitrothion, and S-methyl fenitrothion was detected in both incubates. Significant differences in the rates of formation between any compounds are indicated by nonoverlapping 95% confidence limits. Table 2-2 shows that significantly greater quantities of 3-methyl-4-nitrophenol and desmethyl fenitrothion were formed in the incubates from crabs which were acclimated to 17 ppt seawater than those acclimated to 34 ppt seawater. Significantly greater quantities of fenitrooxon were detected in the incubate from crabs acclimated to higher salinity seawater. In the cytosolic fraction, desmethyl fenitrothion was formed approximately 2.7 times faster in the incubates prepared from crabs which had been acclimated to 17 ppt seawater than in the incubates from crabs which had been acclimated to 34 ppt seawater. In the microsomal fraction, 3-methyl-4-nitrophenol and fenitrooxon were formed approximately 1.4 and 0.5 times as fast, respectively, in incubates prepared from crabs acclimated to 17 ppt seawater than in the incubates from crabs acclimated to 34 ppt seawater.

The data presented in Table 2-3 illustrate that significantly greater quantities of 3-methyl-4-nitrophenol, desmethyl fenitrothion, and fenitrooxon were formed in the incubates from crabs acclimated to and incubated at 22°C than in those incubated at 17°C. Desmethyl fenitrothion was formed approximately 6.7 times faster in the cytosolic fraction at 22°C than at 17°C. In the microsomal fraction, 3-methyl-4-nitrophenol was formed 2.4 times faster and fenitrooxon was formed 5.3 times faster at 22°C than at 17°C.

Fenitrooxon was readily degraded in the microsomal incubates as indicated in Table 2-4. Both enzymatic and nonenzymatic conversion of the oxon to 3-methyl-4-nitrophenol proceeded more rapidly at the higher temperature. Nonenzymatic degradation was more rapid than enzymatic degradation at both 17°C and 22°C.

Table 2-4. Percent fenitrooxon converted to 3-methyl-4-nitrophenol per minute in microsomal incubates

	17°C	22°C
Total	3.1 ± 0.5	5.8 ± 0.6
Nonenzymatic	2.3 ± 0.38	3.1 ± 0.27

The data in Table 2-5 indicate that the addition of SKF-525A to the microsomal incubates resulted in a significant decrease of fenitrooxon formation at both temperatures. However, SKF-525A treatment had no significant effect on the levels of 3-methyl-4-nitrophenol or desmethyl fenitrothion detected.

Figure 2-1 shows the effects of varying GSH concentration on the formation of desmethyl fenitrothion. An increase in GSH concentration in the cytosolic incubate resulted in a proportional increase in both the enzymatic and nonezymatic formation of desmethyl fenitrothion.

Table 2-6 presents the tissue distribution of GSH transferase and aniline hydroxylase activities. It shows that the hepatopancreas is

Table 2-5. The effects of SKF-525A on the *in vitro* metabolism of fenitrothion by sub-cellular fractions prepared from *Callinectes sapidus* hepatopancreas[a]

	nmol formed min^{-1} mg $protein^{-1}$ ± 95 confidence limit			
	17°C		22°C	
	SKF-525A[b]			
Metabolite	-	+	-	+
3-methyl-4-nitrophenol[d]	1.4 ± 0.06	1.3 ± 0.11	3.4 ± 0.35	3.17 ± 0.40
fenitrooxon[d]	0.044 ± 0.004	- [c]	0.21 ± 0.018	- [c]
desmethyl fenitrothion[e]	0.51 ± 0.06	0.55 ± 0.09	2.9 ± 0.51	2.6 ± 0.40

[a] Same conditions, precision, and comparable control values as in Table 2-3.

[b] [SKF-525A] = 30 mM when present.

[c] No fenitrooxon was detected.

[d] Metabolite detected in microsomal fraction.

[e] Metabolite detected in cytosolic fraction.

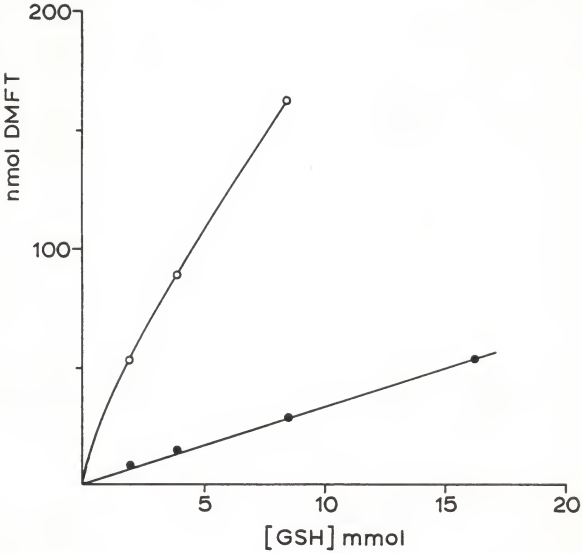

Figure 2-1. The effects of varying glutathione (GSH) concentration on the formation of desmethyl fenitrothion. o = enzymatic product formation; ● = non-enzymatic product formation.

Table 2-6. Tissue distribution of glutathione S-transferase and aniline hydroxylase activity[a]

Organ	gm Wet Weight/3 Crabs[b]	mg Cytosolic Protein/ gm Tissue	mg Microsomal Protein/ gm Tissue	Specific Activity[c] GSH-Transferase	Total GSH-Transferase Units[d]/Organ	Specific Activity[c] Aniline Hydroxylase	Total Aniline Hydroxylase Units[d]/Organ
Heart	4.7	1.4	0.27	200 ± 17.6	1316 ± 169	0.071 ± 0.020	0.090 ± 0.025
Stomach	5.0	2.6	0.32	240 ± 6.0	360 ± 77	0.292 ± 0.142	0.467 ± 0.227
Gills	17.6	4.1	0.52	94 ± 5.0	6783 ± 360	0.197 ± 0.090	1.803 ± 0.824
Hepato-pancreas	25.6	7.1	1.4	220 ± 19.1	40269 ± 3996	0.018 ± 0.010	0.876 ± 0.486
Testis	1.3	2.9	0.08	280 ± 21.9	892 ± 70	0.038 ± 0.019	0.004 ± 0.002
Muscle	ND	2.7	0.14	86 ± 10.1	ND	0.005 ± 0.0004	ND
Hemolymph	ND	2.8	0.4	27 ± 3.8	ND	ND	ND

[a] Mean ± 95% confidence limit from 3 replicates. Confidence limits for all weights and protein determination <10% of listed mean values. Total blood volume and muscle mass was not determined (ND).

[b] Mean weight of 3 crabs used was 422 ± 14 gm.

[c] Specific activity = units x mg^{-1} cytosolic or microsomal protein.

[d] Units = nmol substrate conjugated x min^{-1}.

the largest organ in C. sapidus, its mass comprising approximately 6.1% of the total wet body mass. The gills are the second most massive organ. Of all the organs analyzed, the hepatopancreas had the highest concentration of cytosolic and microsomal protein. The data in Table 2-7 show that hepatopancreas microsomal protein contains 0.33 nmol cytochrome P-450 per mg of microsomal protein. Table 2-6 indicates that the testis had the highest specific GSH-transferase activity, though the levels detected in the heart, stomach and hepatopancreas were comparable to those found in the testis. The levels of GSH-transferase activity detected in the hepatopancreas were an order of magnitude greater than those detected in any other organ. The order of total GSH-transferase activity was hepatopancreas >>> gills > stomach > heart > testis.

Table 2-7. Composition of subcellular fractions prepared from Callinectes sapidus hepatopancreas[a]

mg Hepatopancreas/ gm Body Weight	mg Cytosolic Protein/gm Hepatopancreas	mg Microsomal Protein/gm Hepatopancreas	nmol Cytochrome P-450/mg Microsomal Protein
63 (8.7)	6.9 (1.0)	1.9 (.21)	0.33 (.11)

[a] Mean (SD) of 3 pools of 5 crabs.

Aniline hydroxylase specific activity was highest in the stomach and gills. The level of total aniline hydroxylase activity/organ was found to be gills ≈ hepatopancreas ≈ stomach > heart > testis.

Discussion

The data presented in chapter I indicated that fenitrothion was more toxic to C. sapidus at 22°C than at 17°C and at a salinity of 34 ppt than at 17 ppt. The data presented in Tables 2-2 and 2-3 suggest that differences in the rates of metabolism at these salinities may contribute to this toxicity differential. Crabs acclimated to the lower salinity produced 3-methyl-4-nitrophenol and desmethyl fenitrothion at a faster rate than those acclimated to higher salinities. Both of these metabolites are probably less toxic to C. sapidus as they cannot inhibit acetylcholinesterase (Miyamoto et al., 1978), are more water soluble and more readily excreted than the parent compound. Furthermore, the presence of the hydroxyl functional group in those two metabolites lends itself to conjugation with endogenous water soluble substrates (e.g., glycoside, sulfate) and subsequent excretion. The rate of formation of the toxic acetylcholinesterase inhibitor, fenitrooxon, was greater in the microsomal incubate from crabs acclimated to higher salinity seawater. These data suggest that the Callinectes which have been acclimated to lower salinities may be more efficient in detoxifying fenitrothion and less efficient in forming the toxic oxon than those acclimated to higher salinity waters. These two effects are additive

and are probably at least partially responsible for the decrease in toxicity observed in crabs acclimated to lower salinity water.

Greater rates of formation for these three metabolites were detected in subcellular fractions from crabs acclimated to and incubated at 22°C than in those at 17°C. These data suggest that the extent to which the increased rate of formation of fenitrooxon contributes to toxicity is not compensated for by the increased rate of detoxification as indicated by an increase in the rate of 3-methyl-4-nitrophenol and desmethyl fenitrothion formation. When fenitrooxon was incubated with the microsomal fraction (Table 2-4) approximately 3.1% and 5.8% of fenitrooxon were degraded per minute at 17°C and 22°C, respectively. This observed instability of fenitrooxon suggests that the levels of fenitrooxon formed _in vitro_ were greater than those detected. Thus the difference in the quantity of oxon formed in the higher and lower temperature incubates may also be greater than these experiments indicated. The primary metabolite of fenitrooxon detected in the cytosolic incubate was 3-methyl-4-nitrophenol. Nonenzymatic hydrolysis of fenitrooxon occurred more rapidly than enzymatic metabolism did at both temperatures.

The addition of SKF-525A to the microsomal incubate caused the concentration of fenitrooxon to fall below the limit of detection. No significant difference in the concentration of 3-methyl-4-nitrophenol was noted between the SKF-525A treated and the nontreated incubates. This suggests that conversion of fenitrothion to fenitrooxon is mediated by a cytochrome P-450 monooxygenase. Ugati et al. (1985) reported an increase in both 3-methyl-4-nitrophenol and fenitrooxon in

microsomal incubates prepared from two strains of the housefly, Musca domestica, after increasing amounts of NADPH. They concluded that the formation of both of these metabolites is catalyzed by a cytochrome P-450 monooxygenase system. Conner and Lee (1982) reported the occurrence of two cytochrome P-450 monooxygenases in Callinectes, as well as several other varieties which can be induced by treatment with polychlorinated biphenyls. It is possible that the formation of fenitrooxon and 3-methyl-4-nitrophenol are catalyzed by two different cytochrome P-450 monooxygenases and that only the form responsible for the production of fenitrooxon is inhibited by SKF-525A. The level of cytochrome P-450 in C. sapidus hepatopancreas was found to be 0.33± 0.11 nmol/mg protein. This is similar to the value of 0.18±0.08 reported by James et al. (1979b), and about five times greater than the value of 0.06±0.03 nmol/mg protein reported by Conner and Lee (1982) for C. sapidus.

In many species, dealkylation reactions are catalyzed by cytochrome P-450 monooxygenases (Neal, 1980). In these experiments, however, desmethyl fenitrothion was not formed enzymatically by the microsomal fraction, and the addition of SKF-525A to the cytosolic fraction had no effect on the rate of desmethyl fenitrothion formation. These findings suggest that desmethyl fenitrothion production in Callinectes is not catalyzed by a cytochrome P-450 monooxygenase. Figure 2-1 indicates that the rate of enzymatic formation of desmethyl fenitrothion proportionately increased with increasing amounts of GSH in the cytosolic incubate. This suggests

desmethyl fenitrothion formation in Callinectes is catalyzed by GSH-transferase.

The tissue distribution of GSH-transferase activity, and of aniline hydroxylase activity suggests that the hepatopancreas is not the only organ possessing detectable levels of enzyme activity, and probably not the only organ capable of metabolizing fenitrothion. While the specific activity of GSH-transferase in the hepatopancreas was comparable to that of several other organs, the hepatopancreas possessed the greatest total GSH-transferase activity due to the fact that it is the largest organ. The gills also displayed a significant amount of GSH-transferase activity, and in an in vivo situation, may be responsible for a significant amount of fenitrothion metabolism, especially since they are continually filtering the surrounding medium and are a major site of uptake for a number of lipophilic xenobiotics (James et al., 1979b).

The in vitro specific aniline hydroxylase activity was quite low for the hepatopancreas, especially in comparison with the gills and stomach. This may be attributed to the presence of endogenous inhibitors which are released during the homogenization procedure. The occurrence of inhibitors of cytochrome P-450 monooxygenase activity inhibitors has been reported for a variety of substrates in microsomes prepared from the spiny lobster (James et al., 1979b) as well as for the blue crab (Bihari et al., 1984). When added to fish liver microsomes, spiny lobster microsomes inhibited cytochrome P-450 mixed function oxygenase and cytochrome P-450 reductase activities (James et al., 1979b). Studies on the in vivo metabolism of

benzopyrene by the spiny lobster (Little et al., 1985) and blue crabs (Lee et al., 1976) suggest that the hepatopancreas is the major site of metabolism even though only low levels of metabolism were detected in microsomal fractions prepared from the hepatopancreases of these animals. Thus, the hepatopancreas probably plays a much greater role in metabolizing fenitrothion *in vivo* than these *in vitro* studies indicate. Total aniline hydroxylase activity was significant in the gills and stomach as well as in the hepatopancreas, indicating that these organs may play an important role in the metabolism of xenobiotics *in vivo*.

While differences in xenobiotic metabolizing enzyme activity probably contribute to the increased *in vivo* toxicity of fenitrothion to *Callinectes sapidus* at the higher salinity and temperature, one cannot rule out the influences of uptake, absorption, and excretion on this toxicity differential. *In vivo* studies necessary to determine the influence of these factors on toxicity are addressed in Chapter III. These *in vivo* experiments require a radiolabeled substrate due to the low application levels mandated by the extreme *in vivo* toxicity of fenitrothion to *Callinectes sapidus*.

CHAPTER III
THE EFFECTS OF TEMPERATURE AND SALINITY ON THE *IN VIVO* METABOLISM OF FENITROTHION BY *CALLINECTES SAPIDUS*

Materials and Methods

Animals

Male intermolt *Callinectes* which weighed between 150 and 175 g were obtained, transported, and acclimated for 2 weeks to either 22°C, 34 ppt (parts per thousand); 22°C, 17 ppt; or 17°C, 34 ppt seawater as described previously.

Chemicals

Fenitrothion, fenitrooxon [O,O-dimethyl-O-(3-methyl-4-nitrophenyl) phosphate], S-methyl fenitrothion [O,S-dimethyl-O-(3-methyl-4-nitrophenyl) phosphate], amino fenitrothion [O,O-dimethyl-O-(3-methyl-4-aminophenyl)phosphorothioate], desmethyl fenitrothion [O-methyl-O-hydrogen-O-(3-methyl-4-nitrophenyl)phosphorothioate], and 3-methyl-4-nitrophenol were synthesized or obtained as reported in Chapter II. Desmethyl fenitrooxon [O-methyl-O-hydrogen-O-(3-methyl-4-nitrophenyl)phosphate] was synthesized by the method of Hollingsworth (1966). Radiolabelled ^{14}C-fenitrothion was synthesized from 56 mCi/mmol ^{14}C-ring labelled 3-methylphenol (Amersham) by a method based on one reported by Hollingsworth (1966). Starting material (0.5 mg) was diluted with 30 mg unlabelled m-cresol (Aldrich) and then reacted with 41 mg $NaNO_2$ (Aldrich) in the presence of 4 ml concentrated HCl

to convert it to its para nitroso analog. The nitroso group was then oxidized to a nitro group with 30% H_2O_2 (5 ml), glacial acetic acid (4 ml), and 140 mg ammonium molybdate (Fisher). The resulting 3-methyl-4-nitrophenol was recrystallized from water and then dissolved in saturated KOH/methanol. Phenol was precipitated by the addition of cold ethyl ether, and reacted with 50 mg dimethylphosphorochloridate (Aldrich) by refluxing in dry acetone for 5 hours. This reaction mixture was dissolved in benzene, washed twice with water, and concentrated by rotary evaporation in vacuo. The ^{14}C-fenitrothion was cleaned up by thin layer chromatography (TLC) on 20x20 cm 0.25 mm Merck TLC plates developed in acetone:hexane (25:75). The overall yield was approximately 25% and the product was shown to be greater than 94% pure as determined by GC-FID. Radiochemical purity was approximately 95% as determined by thin layer chromatography (TLC) and liquid scintillation counting (LSC). This synthesized ^{14}C-fenitrothion had a specific activity of 234 µCi/mMol.

Dose and Administration

Seawater was obtained from the Whitney Marine Laboratory, Marineland, FL, and diluted to the desired salinity with deionized water. Beakers (4 liters) containing submersible heaters were aerated by pumping air through disposable glass pipettes and were filled with 3700 ml of 17 ppt or 34 ppt seawater so that each beaker represented one of the following conditions: 22°C, 34 ppt; 22°C, 17 ppt; or 17°C, 34 ppt. One crab (approximately 175 gm) which had been previously acclimated to the appropriate salinity and temperature was placed in each beaker. A working solution containing 215 µg fenitrothion/ml

ethanol was prepared and 90 μl aliquots were added to each beaker to give a final concentration of 5.2 μg fenitrothion/liter (18.7 nM).

Uptake and Metabolism Studies

The seawater was assayed for fenitrothion and its metabolites by removing duplicate 200 ml aliquots of seawater from each beaker at 0, 24, and 72 hours. Each aliquot was extracted twice with 200 ml ethyl acetate, adjusted to pH 1 with 3 N HCl, and then extracted again. Extracts from the same sample of seawater were combined, dried over Na_2SO_4, transferred to round bottom flasks, and reduced to about 1 ml by rotary evaporation *in vacuo* at 35°C. They were then transferred to centrifuge tubes by rinsing the flask with ethyl ether, and then concentrated to 1 ml under N_2 at room temperature. These concentrates were then applied to 20x20 cm fluorescent 0.25 mm TLC plates (E. Merk) and developed in toluene:ethyl acetate:isopropanol:acetic acid (8:12:5:3). Duplicates were developed in benzene:ethyl acetate (15:85). Standards were also applied to each plate. Radioactivity was located on the TLC plates by autoradiography using Kodak SB-5 X-ray film and an exposure period of 3 weeks. The identities of the radioactive compounds were determined by comparing the R_f values with those of the standards which were co-chromatographed on each plate. The silica gel from the regions containing radioactivity was scraped into LSC vials, and 15 ml Aquasol II (New England Nuclear) and 1.5 ml water were added to each vial. Radioactivity was quantitated by LSC in a Searle Analytic 92 liquid scintillation system. Corrections were made for counting efficiency as determined from a quench curve. Background values were determined by counting samples containing 5 ml

of untreated seawater and 15 ml Aquasol II. Control values were determined by an identical procedure except that no animals were placed in the beakers. Microbiological controls were run by placing the crabs in the beakers for 3 days, and then removing them prior to the addition of fenitrothion.

The presence of conjugated metabolites (i.e., glycosides, sulfates) in the seawater was determined by a similar set of experiments except that 50 ml aliquots of seawater were treated at pH 5 with 1.0 ml of 2.5 M sodium acetate buffer, either 0.7 mg glucuronidase (Type H1, Sigma) and 190 mg of D-saccharic acid 1,4 lactone (Sigma) to inhibit sulfatase activity or 1.0 mg sulfatase (Type H1, Sigma). Only buffer was added to the controls. These glucuronidase and sulfatase enzyme concentrations were capable of hydrolyzing 0.3 μmoles of 4-nitrophenyl glucuronide or 4-nitrophenyl sulfate per hour at 37°C and pH 5. The enzyme treated seawater and the controls were then incubated for 30 minutes at 37°C in a shaking water bath. Enzyme treated incubates, controls, and a set of non-incubated controls were adjusted to pH 3 with 1.0 N HCl and extracted in duplicate with 50 ml ethyl acetate. The appropriate extracts were pooled, dried, reduced to approximately 1 ml, and assayed for radioactivity by LSC.

The disposition of fenitrothion based radioactivity was determined by exposing the crabs to fenitrothion as described previously. Crabs were removed at 0, 24, 48, and 72 hours, placed on ice for 10 minutes, and then dissected. The gills, hepatopancreas, stomach, testis, heart, and muscle from the chelipeds were then freeze

dried. Radioactivity was determined following catalytic oxidation of tissue samples weighing approximately 500 mg in a model 3306 Packard Tri-Carb Sample Oxidizer using 12 ml Permaflour V liquid scintillation cocktail and 6 ml Carbo-Sorb carbon dioxide absorber (Packard) per sample. A combustion time of 1.5 minutes per sample was used. Expected recovery was determined by oxidizing a set of ^{14}C-fenitrothion spiked freeze-dried tissues. Radioactivity was determined by LSC. Hemolymph (1 ml) was obtained from the cheliped joints, combined with Aquasol II, and monitored for radioactivity by LSC.

Hepatopancreatic metabolism was determined by a slight modification of the method of Kobayashi et al. (1985). Following exposure to ^{14}C-fenitrothion, the hepatopancreas was removed, combined with 1 vol of 0.1 M acetate buffer (pH 5) and 10 µg of each of the nonradioactive metabolite standards, and homogenized in a teflon-glass tissue grinder. This homogenate was extracted in triplicate with two volumes of benzene:ethanol (9:1). The pH of this homogenate was adjusted to 1.0 with 6 N HCl and was extracted in triplicate again. Extracts from the same homogenate were pooled, dried, concentrated, and analyzed by TLC/autoradiography as previously described. The remaining aqueous layer was adjusted to pH 5 with 3 N NaOH, enzymatically treated, and analyzed for conjugated metabolites as described previously.

Results

Table 3-1 shows the distribution of ^{14}C radioactivity with respect to the major tissues in Callinectes. These data, reported as fenitrothion equivalents, represent unchanged fenitrothion and/or metabolites of fenitrothion. The chemical composition of the radioactivity was not determined in each organ. There were no statistically significant differences in the levels of fenitrothion derived radioactivity for the comparable organs from crabs exposed to the various salinity and temperature parameters, as indicated by overlapping means ± standard deviation. Thus, Table 3-1 gives only

Table 3-1. Tissue and fluid distribution of fenitrothion derived radioactivity (pmol fenitrothion eq) in Callinectes sapidus[a]

Tissue	24 Hours		72 Hours	
	eq/g or ml	eq/organ	eq/g or ml	eq/organ
Heart	144 (31)[b]	71 (15)	294 (23)	144 (11)
Stomach	538 (40)	209 (16)	788 (49)	307 (19)
Gills	336 (38)	267 (30)	331 (40)	263 (32)
Hepatopancreas	677 (40)	1834 (108)	1204 (42)	3264 (114)
Testis	163 (0)	115 (0)	265 (10)	187 (7)
Muscle	69 (19)	ND[c]	105 (12)	ND[c]
Hemolymph	33 (2)	ND[c]	29 (10)	ND[c]
Shell	93 (17)	1655 (302)	123 (14)	2189 (250)

[a] Callinectes exposed to 5.2 ppb fenitrothion at 22°C, 34 ppt.

[b] Mean from 2 replicate animals (standard deviation).

[c] Not determined.

the data from crabs exposed to fenitrothion in 34 ppt, 22°C seawater. These data indicate that there were differences between the levels of radioactivity in different organs at both 24 and 72 hours. Of the organs tested, the hepatopancreas, stomach, and gills had the highest amounts of radioactivity per gram dry weight. Due to its large mass, the hepatopancreas had the greatest amount of radioactivity. With the exception of the hemolymph and gills the levels of fenitrothion derived radioactivity in all the tissues which were analyzed were higher at 72 hours than at 24 hours.

Table 3-2 shows the TLC R_f values for fenitrothion and its metabolites. The array of metabolites excreted by fenitrothion

Table 3-2. Thin layer chromatography R_f values of fenitrothion and its metabolites

	R_f Values in Solvent Systems[a]	
Compound	A	B
fenitrothion	0.82	0.74
aminofenitrothion	0.72	0.63
fenitrooxon	0.67	0.50
3-methyl-4-nitrophenol	0.46	0.38
3-methyl-4-aminophenol	0.35	0.30
S-methylfenitrothion	0.70	0.57
desmethyl fenitrothion	0.18	0.00
desmethyl fenitrooxon	0.09	0.00

[a] A = toluene:ethyl acetate:isopropanol:acetic acid (8:12:5:3); B = benzene:ethyl acetate (15:85).

exposed Callinectes is also listed in Tables 3-3 and 3-4. Although the quantity of radioactivity in the whole animal was not calculated, it is presumed that the remainder of the initial dose is in the crab.

The data presented in Table 3-4 indicate that Callinectes metabolizes fenitrothion to desmethyl fenitrothion, amino fenitrothion, fenitrooxon, 3-methyl-4-nitrophenol and desmethyl fenitrooxon. The metabolite 3-methyl-4-nitrophenol was detected at higher concentrations than any other metabolites and quantitatively appears to be the major metabolite of fenitrothion. There was a greater level of fenitrooxon excreted into the water by 24 hours in the 34 ppt, 22°C seawater than in the 17 ppt, 22°C seawater. At 72 hours after the initiation of the experiment, greater quantities of desmethyl fenitrothion were detected in the 17 ppt, 22°C seawater than in the 34 ppt, 22°C seawater. At both 24 and 72 hours, the levels of fenitrothion detected were less in the 34 ppt, 22°C seawater than in the 34 ppt, 17°C or 17 ppt, 22°C seawater.

The results in Table 3-3 show that 3-methyl-4-nitrophenol and desmethyl fenitrothion were formed nonenzymatically in the seawater controls. As expected, hydrolysis of fenitrothion proceeded more rapidly at 34 ppt, 22°C than at 34 ppt, 17°C. A difference in salinity from 34 ppt to 17 ppt had no significant effect on the rate of chemical hydrolysis or ratio of hydrolysis products at 22°C (data not shown). Also, no significant differences in these parameters were noted between the controls and the microbiological controls.

Since the hepatopancreas contained higher levels of fenitrothion derived radioactivity than any other organs which were analyzed

Table 3-3. Nonenzymatic degradation of fenitrothion[a,b]

	Time (Hours)							
	17°C				22°C			
Compound	0	24	48	72	0	24	48	72
fenitrothion	1879	1860	1840	1880	1880	1858	1837	1818
3-methyl-4-nitrophenol	0	6.6	13.6	20.6	0	8.2	15.0	24.4
desmethyl fenitrothion	0	11.4	24.4	34.0	0	14.2	26.2	37.6

[a] pmol/100 ml.

[b] Standard deviation <5%. The hydrolysis of fenitrothion was not significantly different at 34 ppt, 22°C than at 17 ppt, 22°C. Results from controls and microbiological controls were not significantly different.

Table 3-4. Fenitrothion metabolites excreted from Callinectes and recovered from seawater[a]

Compound	24 Hours			72 Hours		
	34 ppt/22°C	17 ppt/22°C	34 ppt/17°C	34 ppt/22°C	17 ppt/22°C	34 ppt/17°C
fenitrothion	1184 (32)[b]	1320 (28)	1258 (29)	499 (19)	565 (22)	607 (35)
amino fenitrothion	152 (10)	140 (13)	130 (9)	250 (20)	246 (13)	246 (12)
fenitrooxon	28 (5)	17 (3)	22 (5)	33 (6)	18 (5)	25 (7)
3-methyl-4-nitrophenol	208 (8)	201 (11)	155 (7)	594 (23)	636 (24)	502 (15)
desmethyl fenitrothion	33 (5)	40 (4)	33 (4)	46 (6)	66 (5)	60 (6)
desmethyl fenitrooxon	41 (6)	39 (5)	35 (4)	91 (7)	103 (14)	90 (9)
Percent of initial dose added to seawater	88	94	88	82	87	83

[a] pmol/100 ml.

[b] Means of duplicate analysis from 2 replicates (standard deviation).

Note: All values corrected for nonenzymatic formation of 3-methyl-4-nitrophenol and desmethyl fenitrothion as listed in Table 3-4.

(Table 3-1), it was analyzed to identify the metabolites using crabs which were exposed to ^{14}C labelled fenitrothion in 34 ppt, 22°C seawater. The data presented in Table 3-5 show the levels of fenitrothion and its metabolites detected in hepatopancreas at 24, 48, and 72 hours. All of the metabolites which were detected in the seawater were also detected in the hepatopancreas. In addition,

Table 3-5. Levels of fenitrothion and its metabolites in Callinectes hepatopancreas[a,b]

Compound	Duration of Exposure (Hours) 24	48	72
desmethyl fenitrooxon	79 (5)[c]	148 (12)	279 (35)
desmethyl fenitrothion	78 (7)	92 (6)	85 (5)
3-methyl-4-aminophenol	39 (5)	31 (5)	40 (5)
3-methyl-4-nitrophenol	201 (16)	292 (18)	354 (35)
fenitrooxon	37 (6)	88 (5)	198 (10)
amino fenitrothion	30 (1)	27 (8)	47 (10)
fenitrothion	175 (14)	191 (10)	225 (39)
3-methyl-4-aminophenol glycoside[d]	25 (5)	46 (15)	57 (9)
3-methyl-4-nitrophenol glycoside[d]	16 (6)	19 (7)	22 (4)
3-methyl-4-aminophenol sulfate[e]	10 (5)	19 (7)	31 (5)
3-methyl-4-nitrophenol sulfate[e]	3 (0)	7 (2)	45 (15)

[a] Callinectes exposed to 5.2 ppb fenitrothion at 22°C, 34 ppt.

[b] pmol/g hepatopancreas (wet weight).

[c] mean (standard deviation) from 2 replicate animals.

[d] compound released after treatment with β-glucuronidase.

[e] compound released after treatment with sulfatase.

3-methyl-4-aminophenol, and the glycoside and sulfate conjugates of both 3-methyl-4-aminophenol and 3-methyl-4-nitrophenol were detected in the hepatopancreas. In general, the levels of all of these radiolabelled compounds in the hepatopancreas increased with time. At 24 hours, higher concentrations of fenitrothion and 3-methyl-4-nitrophenol were found in the hepatopancreas than of the other metabolites which were detected. At 72 hours, more desmethyl fenitrooxon and 3-methyl-4-nitrophenol were detected in the hepatopancreas than fenitrothion or any other metabolites. The levels of fenitrooxon at 72 hours were much greater than those detected at 24 hours.

Discussion

Table 3-1 shows that fenitrothion and/or its metabolites are distributed throughout the crab by 24 hours after exposure. It appears that the levels of the compounds had equilibrated in the gills and hemolymph by this time as the amount of radioactivity had not changed substantially by 72 hours. The ratio of the metabolites in these organs may be different at these two sampling times. The levels of fenitrothion equivalents in all the other organs which were analyzed were greater at 72 hours than at 24 hours.

Lower concentrations of fenitrothion were detected in the 34 ppt, 22°C seawater (Table 3-4) than in either the 34 ppt, 17°C or the 17 ppt, 22°C seawater at both 24 and 72 hours. This suggests that *Callinectes* absorbed the fenitrothion more rapidly at both the higher temperature and the higher salinity. This increased rate of

absorption probably contributes to the increased toxicity previously associated with this higher temperature and higher salinity as reported in chapter I.

The radiolabelled compounds identified in the seawater following exposure to fenitrothion (Table 3-4) suggest that Callinectes are capable of metabolizing fenitrothion to a variety of compounds. They are capable of reducing the nitro to an amino group since aminofenitrothion was detected which is further substantiated by the fact that no reduction was evident in the microbiological controls and that hypoxia was unlikely, due to the level of aeration. The presence of fenitrooxon shows that Callinectes oxidize the phosphorothioate to a phosphate. O-Desmethylation reactions can also occur with both fenitrothion and fenitrooxon as substrates as indicated by the presence of desmethyl fenitrooxon and desmethyl fenitrothion. Callinectes are also capable of metabolizing fenitrothion by cleavage at the P-O-aryl linkage to yield 3-methyl-4-nitrophenol, the most abundant metabolite detected in the seawater. At 24 hours, the levels of fenitrooxon detected in the 34 ppt, 22°C seawater were greater than those detected in the 17 ppt, 22°C seawater. It is generally accepted that this conversion of fenitrothion to fenitrooxon is a toxification reaction, as the oxon is a more potent inhibitor of acetylcholinesterase than is fenitrothion. Inhibition of acetylcholinesterase is accepted as the mode of toxicity of organophosphate insecticides (Corbett, 1974). It seems likely that this greater production of fenitrooxon at 34 ppt than at 17 ppt may also contribute to the previously reported greater toxicity of fenitrothion at the

higher salinity. This salinity dependent effect on the production of fenitrooxon has also been shown to occur *in vitro* in microsomes prepared from *Callinectes* (chapter II).

At 72 hours, lower quantities of desmethyl fenitrothion were detected in the higher salinity seawater. This too compliments the findings in the previously cited *in vitro* studies. The formation of desmethyl fenitrothion, as well as all the other metabolites detected except fenitrooxon, probably is a detoxification reaction for *Callinectes*, as these compounds are more water soluble and thus more easily excreted than the parent compound. Furthermore, these compounds have been shown to be less toxic than fenitrothion to both killifish (*Oryzois latipes*) and daphnia (*Daphnia pulex*) (Miyamoto et al., 1978). These data suggest that at the higher salinity, the additive effect of increased oxon and decreased desmethyl fenitrothion production contributes to the increase in toxicity.

Analysis of the hepatopancreas for the chemical composition of the radioactivity (Table 3-5) yielded all the radiolabelled compounds that were detected in the seawater as well as 3-methyl-4-aminophenol, the sulfate and glycoside conjugates of 3-methyl-4-aminophenol and 3-methyl-4-nitrophenol. These compounds which were detected only in the hepatopancreas were present at lower levels than those compounds which were also detected in the seawater. It is quite likely that 3-methyl-4-aminophenol as well as the conjugated metabolites were present in seawater, but at levels below the 15 pmol/100 ml detection limit. It is also possible that retardation of the excretion of the conjugated metabolites may have occurred due to enterohepatic cycling. Such a

phenomenon has been suggested by James (1982). She found that taurine conjugates of phenylacetic acid, 2,4-D, and 2,4,5-T which were found in the hepatopancreas of the spiny lobster and excreted in the feces were eliminated more slowly than the unmetabolized compounds.

In general, the levels of all the metabolites increased with time of exposure. At 24 hours, the levels of desmethyl fenitrothion and desmethyl fenitrooxon were comparable within the seawater and the hepatopancreas. At 48 and 72 hours, desmethyl fenitrooxon levels exceeded those of desmethyl fenitrothion. At 24-72 hours there was 1.4-2.1 times more desmethyl fenitrooxon than fenitrooxon present while there was only 0.4 times as much desmethyl fenitrothion as fenitrothion present. While the conversion of fenitrooxon to its desmethyl analog appears to be an important reaction, this might not be the only means of detoxification of fenitrooxon by Callinectes. These in vivo experiments do not permit one to determine if 3-methyl-4-nitrophenol is formed from fenitrothion, fenitrooxon or both compounds. In a study of fenitrothion metabolism by flour beetles, Rowlands and Dyte (1972) assayed for the metabolites which lacked the phenolic moiety. They identified both dimethyl phosphorothioate and dimethyl phosphate as well as traces of monomethyl phosphate. This suggests that 3-methyl-4-nitrophenol was formed from both substrates. Since insects are physiologically quite similar to crustaceans, it is likely that Callinectes can also hydrolyze fenitrothion and fenitrooxon to yield 3-methyl-4-nitrophenol. This hypothesis is further supported by in vitro studies in chapter II which showed that 3-methyl-4-nitrophenol was formed from fenitrooxon

in hepatic microsomal incubates which were prepared from Callinectes. It is also likely that enzymatic hydrolysis of desmethyl fenitrothion and desmethyl fenitrooxon make a minor contribution to the levels of 3-methyl-4-nitrocresol as these demethylated compounds inhibit acetylcholinesterase in vitro (Miyamoto et al., 1978). Since this acetylcholinesterase inhibition involves enzymatic hydrolysis of desmethyl compounds, it is possible that the enzymes which hydrolyze fenitrothion and fenitrooxon can also hydrolyze desmethyl fenitrothion and desmethyl fenitrooxon.

The fenitrothion metabolites produced by Callinectes are common for a variety of species and with the exception of the metabolites in which the nitro group was reduced to an amino group, are virtually identical to those reported for rainbow trout (Takimoto and Miyamoto, 1976). Desmethyl fenitrothion, desmethyl fenitrooxon, fenitrooxon, and 3-methyl-4-nitrophenol have all been detected in mammals, fish, and plants which had been exposed to fenitrothion. Amino fenitrothion and 3-methyl-4-aminophenol have been associated with fenitrothion metabolism by mammals and bacteria. Nitroreduction has also been noted in other marine invertebrates, such as the lobster, Homarus americanus (Emamlouk and Gessner, 1976) and the quahaug, Mercenaria mercenaria (Carlson, 1972). Conjugation of 3-methyl-4-nitrophenol has been reported in birds, mammals, fish, and plants (Miyamoto et al., 1978). Also, there is no evidence suggesting that the conjugation of the desmethyl compounds occur in any species during fenitrothion metabolism, nor that the phenolic methyl group was oxidized by

Callinectes as has been reported in mammalian metabolism (Miyamoto et al., 1976).

Kobayashi et al. (1985) investigated the in vivo metabolism of fenitrothion in tiger shrimp, Penaeus japonicus. At 24 hours, 3-methyl-4-nitrophenol was the major metabolite detected, while fenitrothion, fenitrooxon, desmethyl fenitrooxon, and desmethyl fenitrothion were all detected. The relative ratios of these metabolites to each other in Penaeus are similar to those observed for Callinectes in this study. The other metabolites of fenitrothion which were observed for Callinectes were not reported by Kobayashi's group. However, this group scraped only the regions from the TLC plates which had R_f values corresponding to their 5 authentic standards. Consequently, regions containing low levels of other fenitrothion metabolites might not have been detected.

These experiments indicate that differential rates of absorption contribute to the salinity and temperature dependent differences on the in vivo acute toxicity of fenitrothion to Callinectes. Salinity has also been shown to affect the rates of formation of fenitrooxon and desmethyl fenitrothion, and this too may affect the toxicity of fenitrothion to Callinectes. The tissue distribution of radioactivity and of in vitro glutathione-S-transferase and cytochrome P-450 monooxygenase activities (chapter II) indicate that these metabolic transformations occur in many different organs, but that the hepatopancreas is the major site of fenitrothion metabolism in Callinectes. From metabolites isolated from seawater and hepatopancreas homogenates, it is apparent that Callinectes have the

potential to metabolize fenitrothion via hydrolytic, oxidative, and reductive reactions. Based on these findings as well as the previous *in vitro* studies, the metabolic scheme for the metabolism of fenitrothion by *Callinectes* is proposed in Figure 3-1. The *in vitro* studies suggest that the oxidation of fenitrothion to fenitrooxon is mediated by cytochrome P-450 monooxygenases while the formation of desmethyl fenitrothion and desmethyl fenitrooxon is dependent upon glutathione-S-transferases. With the exception of 3-methyl-4-aminophenol, which could be formed from amino fenitrothion and/or 3-methyl-4-nitrophenol, the precursors for all the other metabolites are evident.

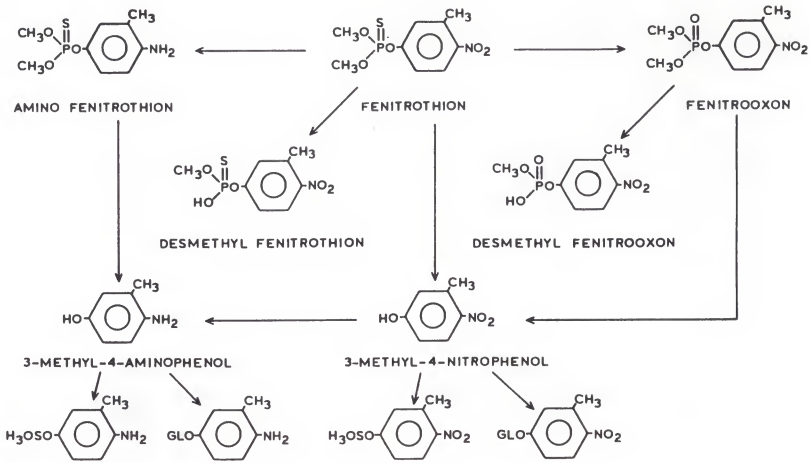

Figure 3-1. Proposed metabolic pathways for fenitrothion in Callinectes sapidus. GL = glycoside.

CHAPTER IV
SUMMARY

The toxicity data presented in chapter I indicate that Callinectes sapidus are extremely sensitive to the acute toxicity of fenitrothion. The 96 hour LC_{50} of 8.6 µg/liter for Callinectes indicates that Callinectes are more resistant to the toxic effects of fenitrothion than are brown shrimp, Penaeus aztecus (48 hr LC_{50} = 2.5 µg/liter); American lobsters, Homacus americanus (48 hr LC_{50} = 1.0 µg/liter) (McLeese, 1976); and tiger shrimp, Penaeus japonicus (24 hr LC_{50} = 1.0 µg/liter) (Kobayashi et al., 1985). The data indicate that marine crustaceans are extremely vulnerable as non-target organisms to the toxic effects of fenitrothion and probably to organophosphate insecticides in general.

Exposure of marine crustaceans to subacutely toxic levels of organophosphate insecticides is even more probable, and this study indicates that exposure to fenitrothion at levels as low as 0.1 µg/liter affects the autotomization response in Callinectes. This might consequently affect the interaction of Callinectes with its environment, and ultimately its survival rate. If acute toxicity parallels subacute toxicity, then one would expect the other referenced species of crustaceans to be more susceptible to the subacutely toxic effects of organophosphates.

Leffler (1974) showed that an abrupt transfer of crabs from high salinity to low salinity seawater resulted in a large increase in metabolic rate. In chapter II, a similar procedure was used to simulate exposure to tidal cycles. It is likely that this increased metabolic rate would lead to an increased rate of absorption of fenitrothion from seawater. Thus, the increased toxicity associated with exposure to fenitrothion and tidal cycles may be due to an increase in the rate of absorption of fenitrothion.

The *in vitro* studies in chapter II showed that greater quantities of 3-methyl-4-nitrophenol, desmethyl fenitrothion, and desmethyl fenitrooxon were detected in the 22°C incubates than in the 17°C incubates. This trend was not apparent in the *in vivo* studies as there were no significant temperature induced differences in the quantities of compounds detected. However, the rate of uptake of fenitrothion by *Callinectes* was greater at the higher temperature. It is possible that the increased rate of absorption is accompanied by higher initial levels of toxic metabolites. By 24 hours, the overall rates of formation of the metabolites might have decreased as a symptom of subacute toxicity. Such a decrease in various enzyme activities and/or energy synthesis has been shown for mussels which had been exposed to subacutely toxic levels of methyl parathion (Moorthy et al., 1985).

These data indicating that an increase in either temperature or salinity increased the toxicity of fenitrothion to *Callinectes* might be used in certain instances to lessen the probability of the occurrence of undesirable nontarget effects due to insecticide

application. This might be accomplished by modifying spraying schedules or parameters to decrease the likelihood of contamination of coastal marine environments when temperature and salinity are at a maximum. Similar application of the tidal cycle data from chapter I suggests that application of organophosphate insecticides in coastal regions might be better done at times when tidal fluctuations are less severe. These data also suggest that the current practice of determining the acute toxicity of chemicals to marine animals at static salinities might result in an underestimate of the hazards of these chemicals in regions where salinity fluctuations periodically occur.

In both the *in vitro* and *in vivo* studies, greater quantities of fenitrooxon and lesser quantities of desmethyl fenitrothion were detected in the higher salinity seawater. In addition, absorption of fenitrothion from seawater was greater in 34 ppt than in 17 ppt seawater. All of these factors probably contribute to the increased toxicity associated with an increase in salinity as determined in chapter I.

Amino fenitrothion and 3-methyl-4-aminophenol were detected in the *in vivo* studies but not the *in vitro* studies. This may be because the *in vitro* conditions may not have been appropriate for *in vitro* nitroreductase activity. Emamlouk and Gessner (1976) detected nitroreductase activity in both the cytosolic and microsomal fraction prepared from the hepatopancreas of the lobster, *Homarus americanus*. In their experiments, the microsomal nitroreductase required NADH while NADPH was required by the cytosolic nitroreductase. In this

study, NADPH was generated in the microsomal but not in the cytosolic fractions. Furthermore, 3-methyl-4-aminophenol was not detected in the in vitro studies as this compound was not retained sufficiently by the reverse phase HPLC column and was not suitable for detection by gas chromatography without subsequent derivitization.

The reason that desmethyl fenitrooxon was not detected during the in vitro metabolism of fenitrothion was that fenitrooxon was formed in the microsomal incubates, while the desmethylation reaction products were detected in the cytosolic incubates. Whole hepatopancreatic homogenates would not have formed desmethyl fenitrooxon from fenitrothion, since the oxon would not have been formed presumably due to the presence of endogenous cytochrome P-450 inhibitors which are released during homogenization.

No whole animal residue levels were determined in this study. One could assume that the average concentration in the animal would be less than that detected in the hepatopancreas, as the hepatopancreas had the highest levels of radioactivity of all the tissues analyzed. If this was the concentration throughout the whole crab, then there would be approximately 34 μg of fenitrothion and/or fenitrothion metabolites per 200 gm crab. The oral LD_{50} for fenitrothion in rats in 800 mg/kg (Berg, 1984).

The LD_{50} for fenitrooxon is about half that of fenitrothion, while the LD_{50} for all the other metabolites which were detected in this study is greater than that of fenitrothion (Miyamoto et al., 1978). It is unlikely that a mammal could consume enough crabs from pesticide contaminated water to suffer acutely toxic effects due to

the residues of organophosphate insecticides and/or their metabolites. Furthermore, fenitrothion, as well as all of its metabolites which were detected in this study have been reported to be nonmutagenic on Salmonella typhimurium TA100 and TA98 regardless of the presence or absence of rat hepatic enzymes (S9 fractions) (Miyamoto et al., 1978).

This study indicates that organophosphate insecticide contamination of coastal regions at levels employed in this study would pose no obvious health threat to the consumer. However, the acute and chronic toxicity of organophosphate insecticides to marine crustaceans is so severe that contamination of our estuaries could drastically affect the ecology of this fragile environment. The effects could be extremely far reaching as estuaries serve as nursery grounds for countless species and are essential to numerous oceanic food chains and life cycles.

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BIOGRAPHICAL SKETCH

John Johnston was born on May 20, 1958, in Jersey City, New Jersey. He received his Bachelor of Science in food science from Rutgers University in January, 1980. He worked as a chemist for National Starch and Chemical in Bridgewater, New Jersey, from January through July, 1980, and came to Gainesville, Florida, in August 1980, to pursue graduate studies in food science and human nutrition. John will receive his Ph.D. from the University of Florida in May 1986.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

M.D. Corbett, Chairman
Professor, Food Science and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

M.O. James
Associate Professor, Medicinal Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

H.A. Moye
Professor, Food Science and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

C-I Wei
Assistant Professor, Food Science and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

W.B. Wheeler
Professor, Food Science and Human Nutrition

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May, 1986

Dean, College of Agriculture

Dean, Graduate School